

DIAGNOSIS AND VACCINATION FOR BOVINE GENITAL CAMPYLOBACTERIOSIS IN
BEEF HEIFERS

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ABSTRACT

Bovine Genital Campylobacteriosis is characterized by early pregnancy loss and temporary infertility in cattle. The purpose of this project was to compare diagnostic approaches to detect *Campylobacter fetus* subsp. *venerealis* and evaluate the efficacy of vaccination for Bovine Genital Campylobacteriosis. This thesis describes the results of two studies that compared different sample preparation methods for bovine vaginal mucus for real-time PCR and assessed a commercial vaccine in preventing infection and reproductive loss.

The first study compared real-time PCR utilizing different bovine vaginal mucus sample preparation techniques to direct culture. The magnetic bead based protocol demonstrated higher sensitivity (48.4%, P=0.02) and lower specificity (78.9%, P=0.01) than the heat lysis protocol which involved an additional dilution step (Sens=29.4%, Spec=88.2%), but did not differ from the heat lysis protocol without sample dilution (Sens=35.0%, P=0.16; Spec=81.1%, P=0.62). The sample preparation method, designed for bovine preputial samples (Chaban et al. 2012. *Can J of Vet Res*; 76: 166), did not work well for vaginal mucus. All modifications of that method and magnetic bead based extraction technique had low sensitivity compared to culture probably due to the biophysical properties of vaginal mucus, which could cause loss of targeted DNA during processing, or repeated sample freezing and thawing. Release of DNA directly from vaginal mucus by a modified heat lysis protocol with consequent real-time PCR could be a promising rapid screening approach after validating on fresh samples.

The second study compared the risk of infection and reproductive failure in heifers, vaccinated with a commercial multivalent vaccine containing *C. fetus* antigen, to heifers vaccinated with a comparable product without *C. fetus*, that were exposed to infected bulls. There was no

significant difference between groups either in risk of *Campylobacter fetus* subsp. *venerealis* isolation ($P>0.17$) or in the proportion of heifers that cultured positive at least once ($P=0.42$), as well as in the median number times of cultured positive samples ($P=0.24$) and the time to first cultured positive ($P=0.67$). There was no difference by treatment in the weekly proportions of heifers diagnosed pregnant by either ultrasound ($P>0.31$) or serum concentration of pregnancy specific protein B ($P>0.31$) during the study, as well as in the time to first pregnancy for heifers ever diagnosed as pregnant ($P=0.30$) and those that remained pregnant at the end of the study ($P=0.70$). Similarly, the difference was not detected by treatment in the proportion of animals, ever detected pregnant during the study ($P=0.57$) and in pregnancy loss rates ($P=0.28$). However, heifers that aborted were 4 times more likely to be cultured positive than those that did not abort ($P=0.01$). Heifers that were not pregnant at the end of the study cultured positive 1.5 times more often than pregnant animals in treatment group ($P=0.04$), while in control group such difference was 4 times ($P=0.01$). Heifers that were not pregnant at the end of the study did not differ by treatment in the number of times cultured positive ($P=0.14$). In this study, the mean concentrations of ELISA antibodies to *C. fetus* after vaccination were more than 2 times higher in treatment group than in control group ($P<0.02$), but vaccination did not significantly reduce infection or improve pregnancy in heifers when exposed to Cf_v-infected bulls.

Sample preparation technique is important for successful real-time PCR; release of DNA directly from a CVM sample by a modified heat lysis protocol was easy to perform and could be promising as a rapid screening approach for Bovine Genital Campylobacteriosis after validating on fresh samples. Vaccinating of heifers with a polyvalent commercial vaccine, containing *Campylobacter fetus* antigen, according to the label, did not significantly reduce infection rate or improve reproductive performance when they were naturally challenged.

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DEDICATION

In the memory of passed away: my parents Mykola and Natalia, and my younger sister Oksana.

To my wife Olena for her continuous patience and understanding, as well as to our little girls Victoria and Kristina. Thanks God, I have a wonderful family.

ПРИСВЯЧУЄТЬСЯ

Пам'яті померлих: моїх батьків Миколи та Наталії, а також моєї молодшої сестри Оксани.

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LIST OF ABBREVIATIONS

BGC	Bovine Genital Campylobacteriosis
Cff	<i>Campylobacter fetus</i> subspecies <i>fetus</i>
Cfv	<i>Campylobacter fetus</i> subspecies <i>venerealis</i>
Cfvi	<i>Campylobacter fetus</i> subspecies <i>venerealis</i> biovar: <i>intermedius</i>
PCR	Polymerase chain reaction
DFAT	Direct fluorescent antibody test
AI	Artificial insemination
CVM	Cervico-vaginal mucus
TLR	Toll-like receptors
NLR	Nod-like receptors
IL	Interleukin
Ig	Immunoglobulin
LPS	Lipopolysaccharide
TEM	Transport and enrichment media
PBS	Phosphate-buffered saline
IFA	Immunofluorescent assay
CSA	Campylobacter selective agar
FITC	Fluorescein isothiocyanate
MAb	Monoclonal antibodies
AGE	Acid glycine extract
WC	Whole cell
CMA	Cervicovaginal mucous agglutination
SA	Serum agglutination
AFLP	Amplified fragment length polymorphism

MLST	Multilocus sequence typing
PFGE	Pulsed field gel electrophoresis
LAMP	Loop mediated isothermal amplification
CTAB	Hexadecyltrimethylammonium bromide
Th cell	T-lymphocyte helper cell
STD	Sexually Transmitted Diseases
APC	Antigen presenting cell
PSPB	Pregnancy Specific Protein B
SQ	Subcutaneous injection

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. GENERAL INTRODUCTION

Reproduction is one of the most important factors influencing profitability in the dairy and beef industries. Reproductive failure can be due to inability to conceive, pregnancy loss, abnormal fetal development and dystocia. Infectious diseases of the reproductive tract are a significant cause of reduced reproductive performance. The most common venereal diseases in cattle include Bovine Genital Campylobacteriosis and trichomonosis.

Bovine Genital Campylobacteriosis (BGC) is caused by the bacterium *Campylobacter fetus* subsp. *venerealis* and characterized by early pregnancy loss and temporary infertility [1, 2]. Bulls are carriers and reservoirs for the disease in the herd where bacteria occupy the glandular crypts of the prepuce [2, 3]. The infection spreads mainly by coitus, causing inflammation in the female genital tract, reproductive failure and large economic loss for the producer [3]. The most common diagnostic methods are culture, direct fluorescent antibody test (DFAT), ELISA, PCR.

The *Campylobacter* genus contains many species including *Campylobacter fetus*, which is divided into two subspecies: *C. fetus* subsp. *venerealis* (previously known as *Vibrio fetus* subsp. *venerealis*) (Cfv) and *C. fetus* subsp. *fetus* (previously known as *Vibrio fetus* subsp. *intestinalis*) (Cff). These organisms differ in their epidemiology and clinical importance; therefore, accurate differentiation is essential, but can be quite difficult if based on traditional biochemical analysis [4–7]. For instance, *C. fetus* subsp. *venerealis* biovar intermedius (Cfvi) can cause BGC, but possesses biochemical features of Cff [8].

Molecular diagnostic methods have been considered to be the most promising for *C. fetus* sub-speciation [9, 10]. Genome size is very similar in both subspecies; however, the genome of most

Cfv strains is about 228 kbp larger than Cff [9] and several genes are subspecies-specific [10]. Conventional PCR, targeting the gene encoding carbon starvation protein A (CstA) (present in both subspecies) and the *parA* gene (present only in Cfv), correlates well with other typing methods when performed directly on colonies from culture media [11, 12].

Successful cultures are influenced by sampling, sample transport conditions, media and environment; therefore, molecular diagnostics may be more robust for direct evaluation of field samples. The correct sample preparation technique is important for a successful PCR assay. A real-time PCR assay has been successfully utilized for testing bull preputial samples [13], but the sample preparation method needs to be validated on bovine vaginal mucus.

Cfv causes endometritis and innate immunity is a vital protection mechanism for exposed female cattle. Inflammatory mediators typically include the interleukin cytokines, tissues necrosis factor, chemokines, interferons and prostaglandins [14]. Acquired immune response includes humoral and cytotoxic immunity. Mucus membranes of the uterus are characterized by a greater IgG than IgA response, depending on the stage of the estrous cycle [15].

Vaccine-induced immunity is usually effective but short-lived and an annual booster is required [16]. The efficacy of vaccines depends on the antigens and adjuvants they contain [17]. Unfortunately, vaccines are not commonly used in the herds most at risk of BGC and there is a relatively small number of peer reviewed publications regarding vaccination for this infection. There is a need to be a better understanding of the efficacy of vaccination in order to make better decisions for a herd's protection.

This review will focus on the etiology, epidemiology, pathogenesis, diagnosis and control of BGC in female cattle.

1.2. ETIOLOGY AND DISTRIBUTION OF BOVINE GENITAL CAMPYLOBACTERIOSIS

Cfv is the etiologic agent for BGC. *Campylobacter* belongs to class Epsilonproteobacteria and the bacteria of this class are highly adapted to mucosal surfaces. These are spiral shaped, slender, curved rods with a corkscrew type of motility due to a polar flagellum on one or both ends, usually 0.2-0.3 x 1.5-5 µm in size, but can be up to 8 µm long [5, 18]. A microaerophilic environment is essential for survival of *C. fetus*; energy for metabolism is obtained from amino acids and intermediates from the tricarboxylic acid cycle [4]. The pathogen is a slow growing, oxidase and catalase positive. Differentiation between *C. fetus* subspecies has been based on the ability of Cff to grow in the presence of 1% glycine and produce H₂S the medium supplemented with 0.02% cysteine-HCl. Cfv do not possess such properties. However, intermediate strains Cfvi have been shown to produce H₂S [8], tolerate 1% glycine [9, 19], and cause BGC [20].

BGC is responsible for fetal losses in cattle and often undetected until pregnancy examination. A high number of animals, exhibiting estrus after the breeding season, could be a diagnostic clue. The economic impact can be severe. For instance, an outbreak in Saskatchewan led to a 70% pregnancy rate in one group and a 24% rate in another group of heifers, bred to different infected bulls [21]. When compared to pregnancy rates of 86, 87 and 88% in three other groups of heifers bred to different uninfected bulls, a 16–62% reduction in pregnancy rates was reported [21].

BGC is distributed worldwide, although there is a lack of reliable data from many countries [3]. The disease has been diagnosed in Europe [22, 23], Asia [24, 25], Africa [26–28], North America [2, 21], South America [29–31], Australia and New Zealand [32–34]. BGC is common in developing countries, where natural breeding is practiced in both dairy and beef herds, while in developed countries it is usually limited to beef herds [35].

C. fetus has been found worldwide in cattle (Table 1.1.), and the reported prevalence varied from 0% to 53% [2, 26, 37, 25–29, 37–46]. The reported prevalence of Cfv was from 0.9% [26] to 17.2% [37] in general, in bovine preputial samples it was 1.8–17.2% [26, 28, 37–40], and in bovine vaginal mucus it was 0.9–3.1% [26, 28]. The prevalence of Cfvi in preputial samples was reported as 12.3% in Nigeria [38] and 17.2% in South Africa [37].

1.3. PATHOGENESIS IN COWS AND HEIFERS

1.3.1. Maintenance of *Campylobacter fetus venerealis* in the bovine female genital tract

While infection results in an asymptomatic carrier state in bulls [47, 48], cows and heifers experience transient infertility [49], associated with inflammation in the reproductive tract [50]. The ability of Cfv to persist in the presence of the host immune response is not fully understood. However, antigenic variation is suggested as a possible mechanism and has been observed in both subspecies of *C. fetus* [51–53].

Many changes happen in the antigens expressed on the bacterial surface during infection. In one study, Cfv was isolated from a human and inoculated into the uterus of a BGC-negative heifer. Isolates obtained from vaginal mucus on a weekly basis exhibited variations in high molecular mass protein profiles when compared to the original inoculum [54]. In another study, Cfv, isolated from a bull twice in a 49-day interval, showed different patterns of surface array proteins [55]. Marked antigenic variation was demonstrated in monthly isolates from the two heifers by agglutination tests. Changes in antibody specificity in cervico-vaginal mucus (CVM) and antigenic variation in the bacteria were proposed as a possible mechanism for prolonged maintenance of Cfv in the reproductive tract in the presence of mucosal antibody, leading to an asymptomatic cervico-vaginal carrier state [53].

1.3.2. Immunity against *Campylobacter fetus*

Protection against pathogenic invaders begins with physical barriers. The vulva, vagina and cervix protect the uterus from ascending infection. Other physical barriers include the epithelium of the vagina and endometrium, basement membranes of ovarian follicles and the zona pellucida of oocytes [14]. Antimicrobial peptides and glycoproteins cover the mucosa to prevent bacteria from reaching the endothelium and neutralize the invader [56].

Cervical mucus is produced by mucus-secreting epithelial cells in the cervix. The mucus comprises two phases: aqueous and gel [57]. The aqueous phase contains low molecular mass compounds that are dissolved in up to 95% water [58]; the gel phase is formed mainly by mucins [57, 59]. Properties of CVM are controlled by estrogen level [57]. Mucus is secreted during the whole cycle, but its volume increases at estrus under estrogenic influence. CVM is a good substrate for Cfv growth, but it also contains immune cells and immunoglobins [60].

Innate immunity is vital to protect the female reproductive tract from microbial infections. Toll-like receptors (TLR) and Nod-like receptors (NLR) are pattern recognition features on host cells that are able to sense pathogens [14]. The TLRs and NLRs bind pathogen-associated molecular patterns (PAMP), found exclusively in prokaryotes [61]. In addition, some substances, such as nucleic acids, ATP and IL1 α , released from damaged or dead cells and known as damage associated molecular patterns (DAMP), are believed to bind pattern recognition receptors as well [reviewed in 14].

There are several kinds of TLRs and NRLs that localize in the different parts of host cells binding different PAMPs. The most important TLRs are TLR1, TLR2, TLR6, functioning to bind microbial lipopeptides, and also TLR4. TLR4 principally binds the cell wall component of

Gram-negative bacteria, such as *Campylobacter*, at the lipopolysaccharide (LPS, endotoxin) [14, 61, 62] and responds by secreting inflammatory mediators, such as cytokine IL6, chemokine IL8 and prostaglandin E₂. Activation of other TLRs leads to production of other mediators of inflammation, such as interferons. NLRs, such as NOD1, NOD2, NLRP3 and NLRC4, are intracytoplasmic receptors of host cells that are able to differentiate exogenous molecules from microbial molecules, which is important in case of intracellular pathogens, not Cfv. NLR activation leads to cleavage of caspase-1 to an active form and production of mature cytokine IL1 β , which is another inflammatory mediator [14, 62]. Inflammatory mediators recruit neutrophils and monocytes to the site of infection [61].

Since many pathogens have evolved strategies to avoid host defenses, adaptive immunity is important. Antibodies are especially important in the defense against extracellular pathogens. It has been hypothesized that IgA acts to immobilize Cfv while the action of IgG is opsonization [63, 64]. Both serum and CVM from virgin heifers, systemically immunized with Cfv bacterin, opsonized Cfv organisms of the same strains as were in bacterin preparation. However, CVM from the animals, locally immunized with the same bacterin, was not opsonic [63]. This suggested that local vaccination most likely did not stimulate release of IgG. In another study, immunoglobulin classes A and G were proven to be essentially equally protective against *Trichomonas foetus* [64]. The same could be true for Cfv, since both organisms cause diseases that are similar in their epidemiology and pathogenesis; however, more research is warranted.

BGC does not significantly induce systemic antibodies during or after infection [48, 65]. Unlike bulls, bovine females develop local antibody responses in the vagina and uterus. Heifers, challenged with Cfv, had agglutinating and immobilizing transient IgM antibodies, followed by persistent IgA and IgG antibodies in vaginal secretions, with predominantly IgG antibodies in

uterine secretions [64]. Since CVM from the locally immunized heifers did not demonstrate opsonic activity in one study [63], it could be suggested that IgA is predominant in vaginal mucosa. Because there is a more robust antibody response in female genital mucosal secretions, Cfv infection is not as persistent in cows as it is in bulls [66–68].

In general, genital IgA are of longer duration than IgG. IgA lasted at least 10 weeks after vaginal vaccination, preventing re-colonization of the uterus in vaginal carriers, immobilizing Cfv; however, the pathogen persists in cervico-vaginal area since opsonization does not occur [63, 64]. The local vaccination against BGC, performed by intra-vaginal-cervical route, most likely elicits increase of IgA in CVM and subsequently prevents colonization of uterine cavity [63]. Systemic vaccination leads to higher level of systemic IgG that consequently are secreted mostly to uterine and in some degree into vagina [64].

Whether evasion of host response or protection is predominant probably depends on the magnitude, specificity and isotype of the immune response, as well as the evasive capabilities of the microbes. Since Cfv is an extracellular pathogen, it is expected that mainly antibody responses are associated with protection. Cell-mediated immunity is also likely to play some role, particularly in vaginal carriers or later in infection where lymphocytes are numerous [52]. Both T-cells and antigen processing cells have been shown to be present in the mucosa; phagocytosis, following opsonization with specific antibodies, effectively killed Cfv [48].

1.3.3. Infertility and pregnancy loss associated with Bovine Genital Campylobacteriosis

Infection with Cfv in bovine females can result in reproductive failure [65, 67–73]. In one older study, 10 days after inoculation Cfv localized in the cervix and vagina and resulted in infertility lasting up to 10 months [71]. In another report, the average conception rate was 40% for the first

service in 106 previously unbred heifers, served by infected bulls; 57% developed BGC and only 21% of these became pregnant, whereas among those that escaped infection the conception rate was 67% [74]. Artificially infected heifers conceived with an average 72-day delay after first service and exposure, compare to 13 days in uninfected animals [75].

The inoculation of viable Cfv into the uterus of cows in the second trimester of pregnancy resulted in abortion in 5–7 days; fetal death happened several days before expulsion, and the level of progesterone in peripheral plasma declined at the time of fetal death. Cows, inoculated during the third trimester, aborted in an average of 13 days and decline of progesterone levels on the day of abortion was similar to that in normal parturition [76]. Similar results were obtained after inoculation into the placental cavities [77]. Decline of progesterone at the time of abortion might be due to placental dysfunction and luteolysis, likely because of the products released from autolysis of the infected fetus [76]. The possibility that Cfv directly acts as a luteolytic factor seems questionable as Cfv prolonged the functional life of the corpus luteum (CL) in sheep in another study [78].

Cfv was located by immunofluorescent technique within the endometrium of experimentally infected heifers and recovered from the lungs, abomasum, and occasionally from the liver, spleen, and brain of aborted fetuses [77]. The most frequent fetal lesions from artificially induced BGC were neutrophilic bronchopneumonia and interstitial pneumonia [79]. When aborted between 129 and 187 days of gestation, fetuses were partially macerated but did not show remarkable lesions, suggesting inability to develop the adequate immune response [77]. Fetuses aborted after 222 days of gestation demonstrated grossly fibrinous peritonitis, pleuritis and hepatitis, accompanied by necrotizing placentitis [77, 79]. Histologic observations included changes of a proliferative nature such as reticuloendothelial hyperplasia and plasma cell

infiltrates in the lamina propria of the tubular organs [77]. Therefore, fetuses, infected in later gestation, resisted to a greater degree, since they developed a greater immune response that can be assumed based on the more prominent gross lesions [77–79].

It has been suggested that Cfv causes infertility in cows by restricting the supply of oxygen to the pre-implantation embryo [80]. However, the effect of Cfv on embryonic development has only been studied in murine embryos [81]. Differentiation and hatching percentages were evaluated from morulae, while hatching, adhesion and expansion percentages were noted from blastocysts, cultured with the addition of *C. fetus*. There was no negative effect on the early embryonic development, but the differentiation percentages were 16% less and hatching percentages were 22% more than in morulae and blastocysts, cultured without *C. fetus* [81].

1.4. DIAGNOSIS

1.4.1. Samples

Sample characteristics

Blood, preputial material, CVM, aborted fetuses and placenta can be used for BGC testing. Serum and CVM are used for immune-based diagnostic methods, while other samples are used for pathogen detection and/or isolation. Preputial samples can be a mixture of different components, such as smegma, blood, epithelial cells, urine and feces, but they are usually not as viscous as CVM. Properties of CVM, unlike preputial samples, depend on the stage of the reproductive cycle when sampling was done [82]. Normal microbiota together with possible contaminants from feces make preputial samples complex to investigate [83, 84], while CVM is usually less contaminated with environmental microorganisms, compared to preputial samples.

However, CVM is also contaminated and the goal of good sampling to reduce the contamination as much as possible [82].

Bovine CVM comprises aqueous and gel phases [57]. The aqueous phase contains soluble low molecular weight non-organic compounds while gel phase contains insoluble high molecular weight organic compounds [57, 59]. Biophysical and chemical properties of CVM may pose a challenge for some diagnostic techniques to perform.

There is an inverse relationship between the dry matter content in the aqueous phase and the degree of crystallization [60]. Decreasing in proportion of salts such as NaCl, KCl and CaCl₂ in the aqueous part of CVM leads to decreasing water content. Such changes cause a decrease in crystallization and a relative increase in other salt components in the dry residue [85, 86]. The gel phase of CVM is responsible for rheological properties of vaginal mucus [59].

Dry matter concentration and crystallization patterns of bovine CVM, as well as its viscosity vary during the estrous cycle and early pregnancy [57, 85]. Dry matter concentration reached a minimum value on the day of estrus and a maximum value at the mid-cycle [85], while crystallization increased on the day of estrus [82, 85, 86]. Dry matter concentration of CVM increased from the day of estrus to the 19th day of gestation, while crystallization decreased during this period [85]. During the pre-ovulatory period of estrus cycle CVM is less viscous and more hydrated, making easier posterior movement of spermatozoa [57]. In the luteal phase the viscosity increases preventing migration of sperms [59].

Therefore, whenever CVM is collected it appears “gelly” due to either high viscosity or crystallization. Such appearance could cause DNA loss with pellet when samples are prepared for real-time PCR [57, 59, 82, 85, 86].

Sample collection and transport

Aspiration, washing and swabbing of the vaginal cavity can be used to obtain CVM [3]. To the author's knowledge, there are no data comparing aspiration and washing methods for vaginal mucus sampling.

For aspiration, an artificial insemination (AI) pipette, attached to a syringe, is inserted into the vaginal cavity so that the anterior end of the pipette reaches the cervix; suction is applied while moving the pipette gently backwards and forwards [3]. For washing technique, 20–30 ml of PBS is infused into the vagina through an AI pipette, attached to a syringe; the fluid is sucked out and re-infused several times before being collected. Washing fluid may also be collected from the vaginal cavity by inserting and holding a tampon or gauze after PBS infusion inside of the vagina for 5–10 minutes. Obtained samples may be diluted with PBS or placed directly onto transport and enrichment media (TEM) for culture [3].

Under field conditions samples are often in transportation to lab for >24 hours. *C. fetus* is very sensitive to temperature conditions. In some Canadian provinces, such as Saskatchewan, high temperatures in summer and extremely low in winter make sample delivery quite challenging. *Cfv* is a microaerophilic pathogen that requires minimal oxygen for survival; therefore, transport media should provide such conditions.

When culture is used as a diagnostic method it is essential that samples arrive in a laboratory within 6–8 hours of collection [87, 88]. The poor survival of the organism during transport leads to false negative results [6, 44]. Transport media can help to maintain viability, enhance multiplication of *Campylobacter*, and prevent contaminant overgrowth [88]. Several TEM have been described in literature and summarized in Table 1.2.

1.4.2. Culture

Isolation by culture is the most common BGC diagnostic method and considered to be a gold standard test [3]. Poor viability of CfV is one of biggest obstacles in culture-based testing, leading to specific requirements for culture medium and techniques. The bacteria need 85% nitrogen, 10% carbon dioxide and 5% oxygen in the atmosphere to survive and replicate [47]. To prevent other bacterial overgrowth while supporting growth of *Campylobacter*, filtration and/or selective agar must be implemented.

The filtration of samples with a filter of 0.65 µm pore size prior culturing has been recommended to improve isolation rate of CfV [89, 90]. Historically it was performed with positive pressure on the sample [91], but this resulted in obstruction of the filter pores with tissue particles, consequently reducing colony counts by up to 90% [92]. The filtration method has been improved by using passive filtration that allows motile *Campylobacter* to migrate through the filter pores, resulting in 87% sensitivity when Skirrow's agar was used [93].

Selective media are agar-based with inclusion of 5–10% blood and specific combinations of antibiotics [57, 91]; the most common are campylobacter-selective agar (CSA) and Skirrow's agar [94]. CSA contains antibiotics polymixin B, bacitracin, cycloheximide, novobiocin and demonstrated sensitivity of up to 94% when samples were plated within 2–4 hours after collection [88, 92, 94, 95]. Skirrow's agar, containing higher concentrations of polymixin B than CSA agar plus vancomycin and trimethoprim, can be used for isolation of thermophilic *Campylobacter* [96] with up to 68% of sensitivity [88, 94]. CSA demonstrated higher sensitivity than Skirrow's agar possibly due to susceptibility of some CfV strains to polymixin B [94]. Contamination of samples with fungi reduces the available surface for other organisms in the

medium, and cycloheximide in CSA appears to control this, while Skirrow's agar does not contain antifungal agents. However, the addition of amphotericin B and use of passive filtration can control fungal growth [90, 93].

C. fetus are generally catalase positive, do not produce H₂S in media, negative for hippurate hydrolysis, sensitive to cephalothin, do not grow in the presence of a hypertonic solution (such as 3.5% NaCl) and cannot tolerate a temperature above 42⁰C [97]. For subspecies differentiation, the gold standard method has been the culture in the presence of 1% glycine since Cfv is glycine sensitive and Cff is glycine tolerant [3, 90]. However, glycine tolerance can be acquired through mutation or transduction [98], and glycine sensitive strains of Cff have been reported [99]. Cff, unlike Cfv, has been known to produce H₂S [3, 90]. However, Cffi has the ability to produce H₂S [7, 8] and some isolates tolerate 1% glycine [9, 19], which would give false negative results for BGC in the glycine tolerance test. In addition, phenotypic characterization of Cfv can be influenced by the inoculum size and basal media, resulting in poor reproducibility [12, 100].

The combination of filtration and selective agar has been suggested as a highly sensitive technique in spite of possible disadvantages [91]. While there is no ideal single method for identifying Cfv, the best strategy is to combine synergistic approaches [6, 101].

1.4.3. Immune-based methods

Both direct and indirect immunological assays exist for the detection of *C. fetus*. Direct fluorescent antibody test (DFAT) and capture enzyme-linked immunosorbent assay (ELISA) are examples of the direct tests, while MAT and ELISA are examples of indirect assays.

DFAT detects *C. fetus* antigen, labeled with fluorescein isothiocyanate (FITC) [102, 103], and results can be obtained in a couple of hours [43]. However, if a large number of samples need to

be processed, this test is laborious and demands a certain level of expertise to perform. When compared to culture, DFAT had similar or greater sensitivity [43, 102, 103], ranging from 80% to 94% [88, 92, 104], and specificity has been estimated as 89% [43, 104]. In situations where CVM could not be cultured within 6 hours after collection, the DFAT appeared to be more sensitive method than culture [105]. DFAT has not been able to identify subspecies [43, 88, 92, 102–105].

Recently a monoclonal antibody (MAb) capture ELISA was developed and utilized on preputial samples, where four monoclonal IgG antibodies were used against epitopes in the LPS O-antigens of *C. fetus* serotype A and B strains [106]. However, the assay could not differentiate Cfv from Cff since both shared the same serotype A [106, 107]. In the different study, systemic vaccination did not interfere with the IgA ELISA on vaginal mucus samples in cows because only IgG were present in vaginal mucus. Due to possible false reactions, caused by antibody fluctuations in individual cattle, the ELISA is best as a herd test [108]. Repeated collection of CVM samples provoked suspicious and false positive reactions in the cervicovaginal mucus agglutination (CMA) test, likely because minor sample collection trauma caused an increase in plasma-derived agglutinins in the vagina [109].

The ELISA method was adequate for the evaluation of the systemic immune response in vaccinated bulls [110]. Utilizing of the common indirect immunoassays (MAT and ELISA) in cows and heifers could be problematic due to low sensitivity of agglutination assays and subjective results interpretation. Antigen preparation method also matters. Detergent soluble *C. fetus* antigens have shown better results than other antigen preparations in ELISA [111]. In another study, where acid glycine extract (AGE) and whole cell (WC) antibodies were used in ELISA for the detection of Cfv and Cff antibodies in bovine blood sera, AGE antibodies were

more specific than WC antibodies. A cross reaction was detected between subspecies which led the authors to conclude that ELISA could only be useful as a monitoring test [112].

1.4.4. Molecular diagnostic techniques

Molecular diagnostic techniques for BGC are attractive because they are fast and do not require viable Cfv in the sample [9, 11, 113–137]. In addition, molecular methods have been shown in several studies to differentiate Cfv and Cff [9, 11, 99, 113, 114, 116, 118–122, 126, 129].

Described polymerase chain reaction (PCR) assays to detect *Campylobacter fetus venerealis*

Several PCR methods have been developed to differentiate *C. fetus* from other *Campylobacter* species or other bacteria. Many PCR assays targeted the ribosomal RNA operon or the *cpn60* gene [113–117]. In addition to its usefulness at the species level, 16S rRNA gene-based PCR was subsequently included in studies for Cfv detection [113, 114, 116]. Numerical analysis of pulse field gel electrophoresis DNA (PFGE-DNA) profiles was claimed as an effective method for differentiating *C. fetus* subspecies [117]. PFGE could also differentiate subspecies of *C. fetus* based on estimated genome size [9].

A multiplex PCR assay, described by Hum et al. in 1997, has been used worldwide for detection and subspecies discrimination of *C. fetus*. The assay utilizes two pairs of primers: MG3F/MG4R, targeting the carbon starvation protein A gene (*cstA*), common to both subspecies, and VenSF/VenSR targeting the *parA* gene, considered to be exclusively present in Cfv [11]. Agreement with the glycine tolerance test was reported to be 70–100% [117–122].

The VenSF / VenSR primer set has been validated through 15–20 years of testing and considered reliable in the face of new discoveries [13]. However, two *Campylobacter hyointestinalis* isolates

produced false positive results with a Cfv specific assay in one study because *parA* or a close homologue was present in *C. hyointestinalis* isolates [123]. According to recent evidence *parA* gene, located on a transferable genomic island [18], could be potentially spread among *Campylobacter* species. Significant discrepancies were reported between Hum primer-based PCR and phenotypic methods on 17 Cfv isolates in the UK [124] and 40 Cfvi isolates from South Africa [122]. These disagreements might be due to Hum PCR failure or differences in phenotypic test conditions [120, 125]. Therefore, the positive results should be confirmed by additional tests and more research is warranted.

Other PCR tests, targeting the *parA* gene, have been reported, including a 5' Taq nuclease assay [126] and another, in which Hum's Cfv specific primers were adapted to the SYBR Green qPCR platform [13]. These tests employed samples, prepared by heat lysis of direct preputial samples rather than a DNA isolation method, had a detection limit of just a one bacterial cell equivalent per reaction [13], and both were more sensitive in field trials than Hum primers-based conventional multiplex PCR [13] and culture [126]. The interesting feature of qPCR tests is the possibility of Cfv quantification in the sample; however, the lack of standardization remains an issue [13, 84].

ISCfe1 is an insertion element in the Cfv genome. To target ISCfe1 a set of primers has been developed: CVEN-L and CVEN-R2, and PCR results agreed 100% with glycine tolerance testing and 98% with Hum primers based PCR [121]. Yamazaki et al. (2010) developed a loop mediated isothermal amplification (LAMP) assay using the ISCfe1 targeting primers that had 100% agreement with phenotypic profiling in *C. fetus* subspecies differentiation [127]. More recently, TaqMan real-time PCR, based on targeting of ISCfe1, has been developed for Cfv detection [128]. So called primers CampF4/CampR4, targeting ISCfe1 in a SYBR Green based assay,

demonstrated better subspeciation of *C. fetus* than VenSF/VenSR primers on 1071 isolates with sensitivity of 98.7% and specificity of 99.8% [129]. All of these studies have been performed on isolates and there are no data found about direct use of these types of tests on field samples.

Van der Graaf-van Bloois et al. (2013) concluded that none of the published PCR assays were able to identify *C. fetus* strains correctly at a subspecies level [130]. The *C. fetus* species identification demonstrated 100% sensitivity and 100% specificity. Development of subspecies-specific real-time PCR, targeting ISCfe1, failed due to variation of the target insertion sequence and prevalence in other *Campylobacter* species. One of possible reasons could be the presence of inhibitors in the PCR products [130].

A whole genome comparison between Cfv and Cff has been possible since the complete genome sequence of the Cfv type strain (ATCC 19438^T) was determined [131, 132]. Three pathogenicity islands were identified in Cfv, based on the comparison to the Cff strain 82-40 [10]. One of these contained the *parA* gene and several other genes involved in a type IV secretion system (T4SS). T4SS is thought to play a role in protein transmission to host cells [13, 18]. Some of these genes have been previously used as targets for PCR primers. For example, VirB11 gene was used as a target in an assay developed by Moolhuijzen et al. in 2009 [133]. Later, Iraola et al. (2012) successfully utilized these [133] and Hum's [11] primers in their multiplex PCR assay to analyze the abomasal liquid of aborted bovine fetuses without performing pre-enrichment steps [134].

Sample preparation methods for PCR

In molecular diagnostics, sample type and preparation techniques play a significant role in the success of the test. In one study, better efficiency of PCR was obtained from fetal abomasal fluids than from lung tissues [79]. Groff et al. (2010) evaluated PCR on bull prepuce samples,

cow CVM, and abomasum contents of aborted fetuses, prepared with five lysis protocols: thermal, proteinase K, with guanidine isothiocyanate, with DNAzol and with hexadecyltrimethylammonium bromide (CTAB). The CTAB protocol provided the best results: 24% of 277 clinical samples were positive for *C. fetus* using PCR, while only 2.8% were positive by culture [135].

Blood, being a common component of most TEM, can inhibit PCR [136]. In order to combat possible inhibitory effects, multiple-step DNA isolation protocols, such as those based on magnetic beads, have been developed and shown to be effective for preparation of clinical samples for PCR testing [137]. This method offers potential advantages for PCR-based diagnostic testing for BGC where the gel consistence of vaginal mucus presents a particular challenge.

1.5. CONTROL AND PREVENTION

1.5.1. Control strategies

The best method of BGC prevention is utilizing AI instead of natural breeding; however, the fresh semen must be from Cfv-free bulls. AI is more commonly used in dairy than in beef herds. Segregation of potentially infected and uninfected animals in combination with extensive culling practice can also control BGC, but requires meticulous records and biosecurity [138].

Repeated sampling increased the probability of detecting infection: 2–6 repeated samples tested negative usually provided conclusive evidence of true Cfv-free status [31, 95, 139]. One study reported an increase of culture sensitivity from 39% to 95% when 3 repeated samplings were performed [47]. Combining culture and conventional PCR enhanced Cfv detection in bulls, as well as parallel use of DFAT and culture, or using selective media and filtration [140].

In female cattle, convalescent immunity is partially protective. Cows, re-infected within two years of clearing the infection, were protected against reproductive failure but became vaginal carriers; therefore, enhancement of immunity might result in total clearance of infection [141].

Treatment and prevention might help either eliminate disease from the herd or control it at the minimal acceptable level. In one study, 75% of preputial samples were cultured negative after 30 days of treatment with a 20% solution of dimetridazole chlorhydrate, 14.3% were negative after fluoroquinolone treatment and 100% after long action oxytetracycline [142]. However, it is unknown whether subsequent samplings were performed after the end of the trial to be sure that bulls remained negative. Cows are not usually treated for BGC for practical reasons and vaccination has been remained as the main preventive method.

Systemic immunization has been believed to prevent or eliminate CfV infection by inducing IgG antibodies in genital secretions and serum, shortening infection and reducing reproductive losses [48, 143]. Active or passive immunization with CfV could cure a vaginal carrier state in one study [144]. The mechanism of IgG1 transport across the epithelium is not well understood. A lack of IgE might be partially responsible for the delay in appearance of IgG1 in genital secretions [141]. The IgE response depends upon transport of IgG1 to peripheral organs [141].

Convalescent immunity is associated with the mucosal IgA antibodies, transported across the epithelium *via* the Poly-Ig receptor, and is partially protective. Systemic immunization is thought to clear the chronic infection early enough to prevent adverse outcomes for pregnancy [141]. However, neither vaccinated nor control heifers had a significant increase of systemic antibodies in one study, having only a slight increase after the breeding, most likely due to natural stimulus by the infected bull [145].

1.5.2. Vaccination for Bovine Genital Campylobacteriosis

Vaccines that have been used and questions of their efficacy

Vaccination for BGC has been considered as a safe and highly effective treatment and preventive measure [17, 41, 68, 146–151, 153–157]. Relatively few peer-reviewed studies have been performed in order to assess effectiveness of monovalent and polyvalent, commercial and experimental vaccines for bulls [17, 152–154, 156–158] and cows or heifers [68, 145–153, 155, 157]. The methods of vaccination, challenge, results and authors conclusions are summarized in Table 1.3.

Vaccination with monovalent vaccine has been reported in 1960–80-ties as effective method [17, 41, 68, 146–149, 151, 153–157]. In one study, the calving rate of vaccinated beef cows, mated with infected bulls, was 82%, compare to 59% for unvaccinated cows and 84% for cows mated with uninfected bulls [146]. Conversely, the proportion of either calved late or non-pregnant was 18% in vaccinated and 41% in unvaccinated cows [146]. In another study, vaccine, prepared from an Australian isolate of Cfvi was injected into 23 virgin Guernsey heifers while 10 unvaccinated animals served as controls [147]. When challenged intravaginally with Cfvi culture, weekly vaginal swabs demonstrated the infection in 80% of unvaccinated animals, compare to 13% of vaccinated animals [147]. Vaccination of 188 bulls with monovalent vaccine, prepared with local antigen in incomplete Freund's adjuvant, resulted in their non-infected status after natural exposure in infected areas for 1 year [41]. In another trial, vaccination of 46 heifers and 4 bulls with monovalent vaccine, prepared with local strain, resulted in 33% of infection rate in vaccinated heifers, compare to 68% in control group, and none of 4 bulls were infected [157].

The effectiveness of polyvalent vaccines is questionable. In one study, commercial vaccine, formulated with inactivated antigens of *Leptospira interrogans* serovar pomona, *Haemophilus somnus*, Cfv and Cff, viruses of the infectious bovine rinotracheitis and bovine viral diarrhoea in aluminium hydroxide adjuvant, was used to vaccinate heifers that were naturally challenged for 60 days [145]. Vaccinated and control heifers had a poor reproductive performance and did not increment of systemic antibody level [145].

Commercial and experimental dual vaccines, containing *Trichostrongylus axei* and *C. fetus* antigens, were compared to controls. Vaccinated heifers resisted or quickly cleared both pathogens, had a higher pregnancy rate, and a better immune response [150]. The experimental vaccine, containing antigens from local strains of *C. fetus* and *T. axei*, given both subcutaneous (SQ) and intravaginal, was superior to the commercial vaccine [150]. Whether that was due to the fact of using local strains of pathogens when the experimental vaccine was prepared or due to local application of the preparation, remained questionable.

Antigenic difference between local strains and changes in pathogenicity during infection may reduce vaccine effectiveness [68]. This phenomenon was illustrated in one study in which a commercial vaccine was compared to an experimental vaccine and the adjuvant alone. For experimental vaccine cell suspensions of Cff, Cfv and Cfvi, obtained from aborted heifers, were killed by formaldehyde and emulsified with the oleo adjuvant. Natural exposure was for 90 days and half of the heifers in each group were additionally challenged by intra-vaginal inoculation with Cfv. Vaccinated heifers resisted or quickly cleared the infection, had higher pregnancy rates and higher systemic immune responses than control animals [150]. However, one of earlier studies on 321 bulls demonstrated superior protection of monovalent vaccine, when Cff was used as an antigen instead of Cfv [41]. In other hand, the local strain of Cff was utilized and diagnosis

was based on FAT and culture on selective media, making that conclusion doubtful in the modern time, when more advanced diagnostic methods can be implemented.

The factors that influence effectiveness of vaccination are: animal age, frequency of booster vaccination, and adjuvant used. Heifers, vaccinated as yearlings, derived greater protection than those, vaccinated as calves. Two subcutaneous (SQ) injections of a similar vaccine, administered at 11 and 13 months of age gave greater protection than one injection [148]. Vaccines, prepared from the killed Cfv cells, grown on solid medium, gave greater protection than vaccines, prepared from Cfv, cultivated in liquid medium [149].

There have been reports of utilizing monovalent vaccine with a purpose to cure BGC infection. In the 1973 study, 70% of preputial samples cultured negative after the first and other 30% after the booster vaccination of 41 bulls [41]. Other studies suggested that active systemic immunization would be able to eliminate vaginal carrier state in heifers; however, due to small number of animals conclusion could not be made [68, 155]. Later, in 2005 a Cfv bacterin in oil adjuvant was tested on 27 BGC-positive bulls; however, only half of them were cultured pathogen-free after two vaccinations [158]. Therefore, that method was recommended by authors as an additional strategy only [158].

The role of adjuvants

Recombinant or synthetic antigens in modern vaccines are generally less immunogenic than in older style vaccines, produced from live or killed whole organisms [159]. Therefore, in addition to such a relatively weak acquired immune response strong innate immunity must be elicited. Adjuvants are compounds that increase or modulate the intrinsic immunogenicity of an antigen, eliciting strong and long lasting immune responses.

Many recently developed adjuvants act via a TLR-dependent pathway. The initiation of Th cell responses requires 3 signals: 0, 1 or 2 [160]. In general, adjuvants can act on one or each of these signals, having various effector mechanisms (Table 1.4). Mucosal tissues could be protected by the initiation of Th cell responses, boosting Th17 cells that are produced in the presence of IL-23 and characterized by production of IL-17, starting inflammatory response [161].

The majority of BGC vaccines are prepared with oil or aluminum hydroxide adjuvants [16, 17, 41, 145]. It has been suggested that vaccines made with oil adjuvants are the most effective [16, 17, 41]. Alum was able to induce a good antibody response but had little capacity to stimulate cellular immunity. Alum could cause side effects such as sterile abscesses, eosinophilia and myofascitis [reviewed in 161]. Oil emulsions in systemic vaccination against STDs in bulls stimulated strong antibody and cell-mediated responses, but often caused injection site granulomas [159, 161]. However, newer oil adjuvants are much improved and might be used soon [159]. Alum generally induced an immunity of shorter duration than oil adjuvants [162]. Systemic immunization of 16 heifers with two commercial vaccines in aluminium hydroxide adjuvant with 3-week intervals and a subsequent 60-day natural challenge did not generate a significant rise in systemic antibody titers [145]. Bacterins in oil adjuvants have proven effective and provided long protection after a single dose [163].

Polymer-based adjuvants have been developed in the last decade to enhance the immunogenicity of antigens and reduce the autoimmune responses. Given with an antigen, they induced humoral and cellular immune responses, although they did not induce such response on their own. Usually, they work on the principle of depot generation for slow release of the antigen, acting as an immuno-modulator via strong antigen presentation [reviewed in 164]. The polymer-based adjuvant usage in vaccines against bovine STDs remains the potential for the future.

1.6. RESEARCH OBJECTIVES

1. To compare the sensitivity and specificity of a real-time PCR assay, utilizing different sample preparation techniques, to direct culture of bovine vaginal mucus, confirmed by multiplex conventional PCR, for detection of *Campylobacter fetus* subsp. *venerealis*.
2. To compare the proportion of heifers infected with CfV, following natural exposure to infected bulls (assessed by culture and conventional PCR analysis of vaginal mucus) following vaccination with commercial vaccine, containing *C. fetus* antigen, and vaccinated with comparable vaccine without *C. fetus* antigen.
3. To compare the proportion of, and days to pregnancy loss, between heifers vaccinated with commercial vaccine, containing *C. fetus* antigen, or vaccinated with comparable vaccine without *C. fetus* antigen, using trans-rectal ultrasonography and measurement of pregnancy-associated glycoprotein concentration in serum.

Table 1.1. Worldwide prevalence estimates of Bovine Genital Campylobacteriosis

Continent/ Country		N	Samples	Test	Sub- species	Prev %	Reference
Eurasia	Belgium	329	Prepuce samples	DFAT/ culture	NS	12.5	Bouters et al. 1973 [41].
	UK	190	Placentas, aborted fetuses	ELISA/ culture	Cff	40.5	Devenish et al. 2005 [42].
	China	80	Placentas, aborted fetuses	PCR 16SrRNA	NS	0	Yang et al. 2012 [25].
Africa	Namibia	1201	Prepuce samples	PCR 16SrRNA	NS	2.1	Madoroba et al. 2011 [27].
	Nigeria	585	Prepuce samples	Culture	Cfv	2.1	Bawa et al. 1991 [26].
		104	Vaginal mucus			0.9	
		170	Prepuce samples	Culture	Cfv	1.8	Mshelia et al. 2012 [28].
		100	Vaginal mucus			3.1	
	S. Africa	602	Prepuce samples	Culture	Cfvi	12.3	Mai et al. 2013 [38].
		87	Prepuce samples	Culture	Cfvi	17.2	Pefanis et al. 1988 [37].
	Tanzania	1912	Prepuce samples	PCR 16SrRNA	NS	1.8	Madoroba et al. 2011 [27].
		58	Prepuce samples	Culture	Cfv	5.1	Swai et al. 2005 [165].
North America	Canada	41	Prepuce samples	PCR 16SrRNA	NS	2.4	Madoroba et al. 2011 [27].
		67	Prepuce samples	DFAT/ culture	NS	29.8	Ruckerbauer et al. 1974 [43]
		105	Prepuce samples	DFAT/ culture	NS	16	Garcia et al. 1983 [44].
		200	Prepuce samples	DFAT/ culture	NS	0.5	Finlay et al. 1985 [36].
		529	Prepuce samples	ELISA/ culture	Cff	3.4	Devenish et al. 2005 [42].
South America	Argentina	377	Prepuce samples	PCR <i>parA</i>	Cfv	2.9	Chaban et al. 2012 [13]
		6764	Prepuce samples	DFAT	NS	0.9	Fort et al. 2004 [45].
	Brazil	29	Vaginal mucus (buffalo)	DIFT	NS	17	Cipolini et al. 2009 [46].
		327	Prepuce samples	DFAT	NS	52.3	Pelegriin et al. 2002 [29].
Australia	Colombia	146	Prepuce samples	Culture	Cfv	14.9	Griffiths et al. 1984 [39].
	Australia	504	Prepuce samples	Culture	Cfv	4.1	Turnbull et al. 1977 [40].

Table 1.2. Described transport and enrichment media (TEM) for *Campylobacter fetus*

Media name	Developed or first used for Cfv by	Containing	Properties, usage, advantages and issues
Buffered saline	McMillen et al. 2006 [126].	Distilled water, sodium chloride	Can be used when Cfv viability is not required [126] or for culturing when samples are kept slightly warm for a short period of time [13].
Australian TEM	Clark et al. 1978 [166].	Serum, antibiotics, antifungals	Can be utilized for transportation for no longer than 48 hours at 18–37°C [166]. One report suggested Cfv viability for 24 hours [167], another for 96 hours [168].
Weybridge TEM (WTEM)	de Lisle et al. 1982 [167].	Blood, charcoal, antibiotics, antifungals	Keeps viability for about four days at room temperature or refrigerated [167]. Indicated significantly better isolation rate for 4-hour transport compared to other media [88].
Modified Weybridge TEM (mWTEM)	Lender et al. 1990 [169].	Blood, charcoal, antibiotics, antifungals, FBP (Sodium pyruvate, Sodium metabisulphite, Ferrous sulphate)	Maintained the viability for three days and supported their multiplication [169]. Allowed detection when only 10 organisms were inoculated [140]. Proved 50% more efficient than Australian TEM due to less polymixin B, which could inhibit Cfv [92]. Doesn't completely control overgrowth of <i>Pseudomonas</i> spp. [93, 140].
Thomann TEM	Hardwood et al. 2009 [136].	Nutrient broth, FBP, antifungals, reduced mWTEM antibiotics concentration	Promised survival time up to 8 days at 8–37°C would improve diagnostic capabilities in endemic areas [136].

Table 1.3. Summary of vaccine trials for Bovine Genital Campylobacteriosis

Country	Australia		
Vaccine	<u>Monovalent</u> 46 mg of <u>Cfv</u> cells in mineral oil adjuvant	<u>Monovalent commercial</u> , <u>Cfv</u> cells in mineral oil adjuvant	<u>Monovalent commercial</u> , <u>Cfv</u> cells in mineral oil adjuvant
Animals and treatment	4 Aberdeen Angus <u>bulls</u> , 4-6 years old;	334 <u>heifers</u> , 2 years old (Tx*=224, C*=100);	261 <u>heifers</u> , 2 years old (Tx=157, C=104); 70 <u>bulls</u>
Random assignment	No	Yes	Yes
Vaccination route	2 SQ*, 5 weeks interval.	2 SQ, 5 weeks interval	2 SQ, 5 weeks interval.
Challenge	<u>Culture infusion</u> : 6 months PV*; each bull was <u>mated</u> to 3-4 virgin heifers.	<u>Natural exposure</u> PV to non-vaccinated bulls for 28 days	<u>Natural exposure</u> PV to vaccinated bulls for 70 days
Assessment of outcome	Not blinded	Blinded	Blinded
Results	No positive samples from bulls and heifers.	<u>Pregnant</u> : Tx=76%, C=55%; Non-pregnant in anoestrus: Tx=11%, C=6%.	<u>Pregnant</u> : Tx=85%, C=61%; <u>Concluded</u> that vaccination of bulls did not improve pregnancy rate in heifers.
Statistical analysis	No	P-value reported	P-value reported
Conclusion**	Effective	Effective	Heifers: effective; bulls: questionable
Reference	Clark et al, 1968 [17]	Allan and Mutch, 1971 [151]	Allan, 1972 [152]

*Tx-treatment group, C-control group; PV-post vaccination; SQ- sub-cuteniously;

** by authors

Table 1.3. Summary of vaccine trials for Bovine Genital Campylobacteriosis (continued)

Country	Australia		
Vaccine	<u>Monovalent</u> , bacterins: Cfv isolated from infected heifer and grown in solid (A, B) and liquid (C, D) media.	<u>Monovalent</u> , Cfv cells grown in liquid medium, prepared with mineral oil adjuvant.	<u>Monovalent</u> 40 mg of Cfv cells cultured in liquid media
Animals and treatment	125 Hereford <u>heifers</u> , 5 equal groups: A, C – 11-13 months old, B, D – 17-19 months old, E-control.	80 Hereford <u>heifers</u> , 6 groups: A=17, B=17, C=16 (11-13 months old); D=10, E=10, F=10 (5-8 months old). C, F-control.	102 Aberdeen Angus <u>cows</u> , 2 years old; (Tx*=68, C*=34);
Random assignment	Yes	Yes	Yes
Vaccination route	1 SQ *	Groups A,D: 1 SQ; B,E: 2 SQ, 2 months interval	1 SQ
Challenge	<u>Natural exposure</u> PV* for 3 mating seasons. <u>Culture infusion</u> : at the 1 st service.	<u>Natural exposure</u> PV for 60 days at the age of 19-22 months. <u>Culture infusion</u> : within 2 h of the 1 st service.	<u>Natural exposure</u> to carrier bulls 1-2 months PV
Assessment of outcome	Blinded	Blinded	Blinded
Results	<u>Infected</u> : groups: 1 st season: A=2/22, B=1/24, C=7/22, D=0/25, E=15/23; 2 nd season: A=3/16, B=3/17, C=5/12, D=3/17, E=12/14; 3 rd season: A=3/25, B=2/25, C=2/25, D=3/25, E=10/10. <u>Pregnant</u> : A=91%, B=92%, C=77%, D=88%, E=39%; 2 nd season: A=94%, B=100%, C=92%, D=94%, E=43%; 3 rd season: A=91%, B=100%, C=86%, D=75%, E=20%.	<u>Infected</u> : groups: A=7/16, B=5/17, C=12/14, D=8/8, E=7/10, F=10/10; <u>Pregnant</u> : A=69%, B=88%, C=43%, D=50%, E=70%, F=20%;	<u>Calving in time</u> : Tx=82%, C=59%; non-challenged=84%; <u>late calving or non-pregnant</u> : Tx=18%, C=41%, non-challenged=16%.
Statistical analysis	P-value reported	P-value reported	P-value reported
Conclusion**	Effective		Effective
Reference	Clark et al, 1972 [149]	Clark et al, 1972 [148]	Clark et al, 1972 [146]

*Tx-treatment group, C-control group; PV-post vaccination; SQ - sub-cutaneously;

** by authors

Table 1.3. Summary of vaccine trials for Bovine Genital Campylobacteriosis (continued)

Country	Australia		
Vaccine	<u>Monovalent</u> 40 mg of cells cultured in liquid medium (Tx ₁ *– Cf _v , Tx ₂ –Cf _{vi} cells)	<u>Monovalent commercial</u> , Cf _{vi} cells in repository adjuvant	<u>Bivalent commercial</u> , 20 mg Cf _v and Cf _{vi} cells in mineral oil adjuvant
Animals and treatment	80 Hereford <u>heifers</u> , 20-28 months old (Tx ₁ =20, C* ₁ =20) and 20-28 months old (Tx ₂ =17, C ₂ =20); 13 <u>bulls</u> , 14-18 months old (Cf _v vaccine), 7 bulls, 3 years old (Cf _{vi} vaccine)	33 Guensey <u>heifers</u> , 12-14 months old (Tx=23, C=10);	24 Hereford <u>bulls</u> , 2.5-4 years old (Tx=12, C=12);
Random assignment	Yes	No	Yes
Vaccination route	Heifers: 1 SQ *; bulls: 2 SQ, 8 weeks interval.	1 SQ	2 SQ, 6 weeks interval.
Challenge	<u>Culture infusion</u> : of 10 ⁷ -10 ⁸ Cf _{vi} cells for 8 weeks PV*	<u>Culture infusion</u> of 10 ⁸ Cf _{vi} cells into the fornix of vagina, 30 days PV	<u>Culture infusion</u> : 2 years PV all bulls, 3 years PV 9 bulls in each group
Assessment of outcome	Blinded	Not blinded	Not blinded
Results	<u>Infected</u> : Tx ₁ =12/20, C ₁ =20/20, Tx ₂ =2/17, C ₂ =19/20; bulls: 6/13 and 6/7. <u>Pregnant</u> : Tx ₁ =15/20, C ₁ =9/20, Tx ₂ =15/17, C ₂ =16/20;	Significant <u>titers</u> rise (serum agglutination test) in Tx heifers. <u>Cultured positive</u> (weekly swabs): Tx=3/23, C=8/10	<u>Infected</u> : in 2 years Tx=3/12, C=0/12; in 3 years: Tx=4/9, C=7/9; <u>Recommended</u> : bi-annual booster
Statistical analysis	P-value reported	P-value reported	No
Conclusion**	Effective	Effective	Effective
Reference	Clark et al, 1976 [153]	Johns et al, 1977 [147]	Clark et al, 1982 [154]

*Tx-treatment group, C-control group; PV-post vaccination; SQ - sub-cutaneously;

** by authors

Table 1.3. Summary of vaccine trials for Bovine Genital Campylobacteriosis (continued)

Country	USA		
Vaccine	<u>Monovalent</u> 20 mg/ml Cfv cells in incomplete Freud adjuvant	<u>Monovalent</u> ; A. <u>Whole cell vaccine</u> : 20 mg of Cfv cells; B. <u>water extract vaccine</u> : supernatant; C. <u>“ribosomal” vaccine</u> : broken cells.	<u>Monovalent commercial</u> , 41 mg Cfv cells
Animals and treatment	16 virgin Holstein-Friesen <u>heifers</u> (Tx*=8, C*=8 (4-adjuvant only, 4-non vaccinated));	32 virgin Holstein - Friesian <u>heifers</u> (8-vaccine A, 8-vaccine B, 8-vaccine C, 8-saline).	12 Angus <u>bulls</u> , 5 years old (2-non-infected, 10-infected: Tx=6, C=4);
Random assignment	No	No	Yes
Vaccination route	1 SQ *.	2 SQ days 14-24 post-infecting.	2 SQ, 4 weeks interval.
Challenge	<u>Culture infusion</u> 12-24 h after heat observed.	<u>Culture infusion</u> before vaccination	<u>Culture infusion</u> ; <u>Natural exposure</u> to virgin heifers PV (non-infected=6, Tx=18, C=15.)
Assessment of outcome	Not blinded	Not blinded	Not blinded
Results	<u>Agglutination test</u> : titers increased in Tx animals <u>Infected</u> : Tx=2/8 (25-48 days PV*), C=8/8 (48-51 days PV). Active immunization eliminates BGC vaginal carrier state.	<u>Agglutination test</u> : titers increase in vaccinated heifers. <u>Infected</u> : whole cell vaccine=2/8, water extract vaccine=2/8, “ribosomal” vaccine=2/8, saline=8/8.	<u>Tube agglutination test</u> : increase in serum Ab titers in vaccinated bulls. <u>Infected</u> : non-infected=0/2, Tx=0/6, C=4/4; exposed heifers infected: non-infected=0/6, Tx=0/18, C= 12/15.
Statistical analysis	No	Descriptive only	P-value reported
Conclusion**	Effective	Effective	Effective
Reference	Shurig et al, 1975 [68]	Shurig et al, 1978 [155]	Vasquez et al, 1983 [156]

*Tx-treatment group, C-control group; PV-post vaccination; SQ - sub-cuteniously;

** by authors

Table 1.3. Summary of vaccine trials for Bovine Genital Campylobacteriosis (continued)

Country	Jamaica	Brazil	
Vaccine	<u>Monovalent, Cfv</u> (Jamaican strain)	<u>Monovalent</u> , 2.5 ml suspension of <u>Cff</u> cell in incomplete Freud adjuvant	<u>Monovalent</u> , Cfv bacterin with 15% of oil adjuvant added.
Animals and treatment	46 non-pregnant yearling <u>heifers</u> (Tx*=13, C*=13); 4 <u>bulls</u> , 2 years old	321 <u>bulls</u> (41-positive for Cfv, 288-negative for Cfv);	27 Nelore <u>bulls</u> , tested positive by DFAT;
Random assignment	No	No	No
Vaccination route	SQ *	Curative: 2 s/c, 6 weeks interval; preventive: 1 SQ.	curative: 2 SQ, 23 days interval;
Challenge	<u>Natural exposure</u> of <u>bulls</u> to infected <u>heifers</u>	<u>Natural exposure</u> in infected areas for 1 year PV* for 288 negative <u>bulls</u> .	Were tested positive before the trial
Assessment of outcome	Not blinded	Not blinded	Not blinded
Results	<u>Infected</u> heifers: Tx=33%, C=68%; <u>Pregnant</u> : culture negative=72%, culture positive=29% <u>Infected</u> <u>bulls</u> = 0/4	Positive <u>bulls</u> : 30/41-free after first injection, 11/41-free after the second injection. None of negative <u>bulls</u> were infected.	<u>Infected</u> : 15/27-after 1-st vaccination, 12/27-after 2-nd vaccination. <u>Recommendation</u> : as an additional strategy.
Statistical analysis	No	No	P-value reported
Conclusion**	Effective	Effective	Questionable effectiveness
Reference	Eaglesome et al, 1986 [157]	Bouters et al, 1973 [41]	Foscolo et al, 2005 [158]

*Tx-treatment group, C-control group; PV-post vaccination; SQ - sub-cutaneously;

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Table 1.3. Summary of vaccine trials for Bovine Genital Campylobacteriosis (continued)

Country	Argentina	
Vaccine	<u>Polyvalent</u> , Vac A*: <i>L. interrogans</i> , <i>H. somnus</i> , <u>Cfv</u> , <u>Cff</u> , IBRV, BVDV in aluminium hydroxide adjuvant Vac B: same as Vac A, except <i>H. somnus</i> .	<u>Vac A (commercial)</u> : <i>T. foetus</i> , <u>Cfv</u> , <i>L. interrogans</i> ; <u>Vac B (experimental)</u> : <i>T. foetus</i> , <u>Cff</u> , <u>Cfv</u> , <u>Cfvi</u> (from aborted heifers) in oleo adjuvant.
Animals and treatment	24 Aberdeen Angus , Hereford, cross-breed <u>heifers</u> , 27 months old (Vac A=8, Vac B=8, C*=8);	62 Aberdeen Angus Hereford, cross-breed <u>heifers</u> , 18-24 months old (Vac A=21, Vac B=20, C=21);
Random assignment	Yes	Yes
Vaccination route	two SQ *, 3 weeks interval	Vac A: SQ 60 and 30 days before breeding; Vac B: SQ +i/vaginal 30 days before and 15 days after breeding; C: adjuvant, same method as Vac A.
Challenge	<u>Natural exposure</u> PV* to infected bull for 60 days.	<u>Natural exposure</u> to infected bull for 90 days Vac A ₁ =11, Vac B ₁ =10, C ₁ =10; <u>Natural exposure+ culture infusion</u> on day 30 of breeding (Vac A ₂ =10, Vac B ₂ =10, C ₂ =11).
Assessment of outcome	Not blinded	Not blinded
Results	<u>IFAT</u> results: no important <u>titers</u> increase, slight increase after breeding. <u>ELISA</u> results: slight <u>titers</u> increase. Pregnancy: Vac A=2/8, VacB=3/8, C=0/8. Results are limited due to low number of animals.	Significantly higher <u>ELISA titers</u> in Vac B heifers compare to Vac A and C. <u>Infected</u> : Vac A ₁ =7/11, Vac B ₁ =4/10, C ₁ =8/10; Vac A ₂ =3/10, Vac B ₂ =7/10, C ₂ =9/11. <u>Pregnant</u> : Vac A ₁ =4/11, Vac B ₁ =7/10, C ₁ =2/10; Vac A ₂ =5/10, Vac B ₂ =8/10, C ₂ =6/11. Experimental vaccine – faster rise in systemic Ab and better pregnancy rate.
Statistical analysis	Kappa and P-value reported	P-value reported
Conclusion**	Not effective	Effective, experimental – superior effect
Reference	Cobo et al, 2003 [145]	Cobo et al, 2004 [150]

* Vac-treatment group, C-control group; PV-post vaccination; SQ - sub-cuteniously;

** by authors

Table 1.4. Types of vaccine adjuvants and mechanisms of action

Adjuvant type	Characteristics	Examples
Type A	Specific agonist for TLRs. Acts primary on signal 0, indirectly on signal 2, activating APCs and triggering secretion IL-12 [160]. Can act on signal 1 [170].	Monophosphoryl lipid A (MPL)
Type B	Non-specific. Build a depot at the injection site, leading to high local antigen concentration and improving uptake by APC [171].	Aluminium hydroxide, MF59, Liposomal adjuvants, Freud adjuvants, Nanoparticles, Toxin-derived adjuvants
Type C	Enhance signal 2 through interaction with co-stimulatory molecules on APC. Limited clinical use [159].	TGN1412

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2. COMPARISON OF DIFFERENT SAMPLE PREPARATION METHODS FOR BOVINE
VAGINAL MUCUS FOR DETECTION *CAMPYLOBACTER FETUS SUBSP. VENEREALIS*
WITH REAL-TIME PCR

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2.1. ABSTRACT

Introduction — The laboratory diagnosis of Bovine Genital Campylobacteriosis is complicated by the proper identification of *Campylobacter fetus* subspecies. Although culture is still the gold standard, PCR-based methods have been shown to be a good choice for the detection of *Campylobacter fetus* subsp. *venerealis* (Cfv). Successful Cfv detection is dependent on both the PCR assay used and the sample preparation technique.

Objective — To compare Cfv real-time PCR results for bovine vaginal mucus samples, prepared using heat lysis and magnetic bead based sample preparation techniques, to results of direct, selective culture, confirmed by multiplex conventional PCR.

Study design — Cross-sectional study

Animals — 50 crossbred beef heifers

Materials and methods — Vaginal mucus samples, collected weekly from weeks 4–7 of exposure to infected bulls, were cultured, frozen and subsequently processed for real-time PCR. Initially, real-time PCR results from 50 samples, prepared with four variations of a heat lysis protocol, were compared to culture. These were modifications of a previously described method for bovine preputial samples (Chaban et al. 2012. *Can J of Vet Res*; 76: 166). The two heat lysis protocols with the fewest dilution steps generated the most positive and fewest ambiguous test results and were subsequently compared to culture, confirmed by multiplex conventional PCR, and to results of real-time PCR on 200 samples, prepared with a commercial magnetic bead-based extraction kit.

Results — The magnetic bead-based protocol demonstrated higher sensitivity (Sens=48.4%, P=0.02) and lower specificity (Spec=78.9%, P=0.01) than the first heat lysis protocol (Sens=29.4%, Spec=88.2%), but had no significant differences with the second heat lysis protocol (Sens=35.0%, P=0.16; Spec=81.1%, P=0.62).

Conclusions and future research — Overall, the heat lysis sample preparation method, designed for bovine preputial samples, did not work well for vaginal mucus. All modifications of that protocol and magnetic bead-based extraction protocol had low sensitivity, compared to culture under ideal conditions. More research is needed on fresh vaginal mucus samples where PCR and culture are done concurrently.

2.2. INTRODUCTION

Campylobacter fetus subsp. *venerealis* causes venereal infections in cattle, where inflammation in the female genital tract causes abortion, temporary infertility and prolonged estrus cycle. *Campylobacter fetus* is divided into the subspecies *C. fetus* subsp. *venerealis* (Cfv) and *C. fetus* subsp. *fetus* (Cff), and Cfv can have a biovar "intermedius" (Cfvi). Cfv is highly adapted to the genital tract [1–3], while Cff is mainly isolated from the gastrointestinal tract and only sporadically associated with abortions. Cff can also be recovered from the genital tract with no associated clinical symptoms [1]. Therefore, the subspecies differ in their epidemiology and clinical importance, and accurate differentiation is essential but can be difficult.

There are several diagnostic techniques for detection and differentiation of *C. fetus*; however, each has limitations. For successful culture, the organism must be viable. *Campylobacter* requires special transport conditions, and samples need to be cultured soon after collection or placed into transport media. Isolation of Cfv from preputial secretions was achieved with equal success from samples kept in the modified Stuart's transport medium at 4°C to 6°C for 48 hours and from newly collected samples [4].

The Polymerase Chain Reaction (PCR) is a modern diagnostic tool for detecting *C. fetus* and believed to be able to distinguish subspecies. Hum's PCR is based on two PCR primer sets [5]. One primer set is MG3F/MG4R, which amplifies a 960 base pair (bp) [3] or 750 bp fragment [6] of the *C. fetus* carbon starvation protein gene, present in both subspecies. The second primer set is VenSF/VenSR, which amplifies a 142 bp fragment of the *parA* gene in Cfv [7].

A recent study by McGoldrick et al. reported the successful development of two real-time SYBR Green PCR assays that can detect *C. fetus* (by targeting 140 bp of *nahE* gene) and discriminate

Cfv from Cff (by targeting 150 bp of ISCfe-1) from cultured colonies [8]. However, these assays were validated only on isolates, but a technique that could be directly used on bovine preputial and vaginal mucus samples would be more convenient.

It is not only the identification of an appropriate Cfv-specific gene that is important for successful diagnosis, but also the sample preparation protocol. We anticipated that a heat lysis technique, successfully used on preputial scrape samples [7], would work on vaginal mucus, but might require some modification.

There are differences in the consistency and composition of bull preputial and cow vaginal mucus samples. The composition of cervical-vaginal mucus (CVM) is likely less variable than the mixture of smegma, blood, epithelial cells, urine and feces in preputial samples, but is often more viscous. The consistency of vaginal mucus also depends on the stage of reproductive cycle at the sampling time [9].

Extraction of nucleic acids from mucus samples has been improved by using magnetic beads [10]. Magnetic beads offer many benefits, compare to other sample preparation technologies, since the extraction procedure combines effective steps for disrupting samples that are difficult to lyse. As an example, one such nucleic acid isolation methods employs mechanical disruption of samples with zirconia beads in a guanidinium thiocyanate-based solution that rapidly releases nucleic acids while it simultaneously inactivates nucleases in the sample matrix [10, 11]. Samples are then diluted with isopropanol and paramagnetic beads are added. The surface of these beads is modified for binding of nucleic acids. The beads with nucleic acids are immobilized on magnets and washed to remove proteins and other contaminants. A second wash

removes residual binding solution and the nucleic acid is eluted in a small volume of low salt buffer [11].

The objective of the study was to compare Cfv real-time PCR results for samples, prepared using heat lysis and magnetic bead based sample preparation, to results of direct, selective culture of bovine vaginal mucus.

2.3. MATERIALS AND METHODS

2.3.1. Sample collection

All procedures were performed in accordance with the Canadian Council of Animal Care and approved by the University of Saskatchewan Animal Research Ethics Board (protocols #20100061 and #20100077).

Vaginal mucus samples were collected from 50 cross-bred beef heifers in weeks 4, 5, 6, 7 of exposure to two Cfv-infected Black Angus bulls. During the collection, heifers were restrained in a manual squeeze chute. Sampling was performed by aspiration using a 25” pipette^a, connected to a 20 ml syringe. Prior to aspiration, approximately 2 ml of phosphate-buffered saline (PBS) was passed through the pipette into the syringe. The pipette was then placed inside a solid 12” plastic AI sheath protector^b to minimize fecal contamination. Once the aspiration of mucus was complete, the pipette was withdrawn and rinsed into a 4 ml cryovial tube, containing 2 ml of PBS. Cryovials were transported back to the lab in a styrofoam box with warm water bags within 2 h of collection (25 °C).

A portion of each sample was cultured upon arrival to the laboratory, while the rest of samples were frozen at -70 °C for subsequent real-time PCR.

2.3.2. Laboratory testing

Culture of samples, confirmed by multiplex conventional PCR

Upon arrival at the laboratory, an aliquot of 300 µl was spread onto a 0.65 µm mixed cellulose ester membrane filter^c, placed on 5% blood agar plates, equally covering entire surface of the filter. Incubation was done at 37 °C under microaerobic conditions with an integrated chemical packs for rapid waterless and catalyst-free CO₂ generation^d. Plates were examined after 72 h of incubation and colonies consistent with *Campylobacter* morphology were Gram stained. Plates that contained no evidence of *Campylobacter* colonies were incubated for an additional 72 h. Colonies with Gram stain results, consistent in morphology with *Campylobacter*, were subjected to a conventional multiplex PCR using the primers MG3F/MG4R and VenSF/VenSR as previously described [3].

Comparison of sample processing protocols

We recognized that culture followed by Gram-staining and colony morphology, and confirmed by multiplex conventional PCR remains the “gold standard” test from a regulatory standpoint. However, it was found in a previous study that a PCR assay was more sensitive in preputial samples, collected from Cfv-infected bulls [7].

Initially, 50 samples from the week of bull exposure with the biggest proportion of culture positive results were tested using four heat lysis sample preparation protocols (Table 2.1). The procedures were variations of a protocol previously used for bull prepuce samples [7] and originally modified from McMillen et al. (2006) [12]. For an extraction negative control, 200 µl of ultrapure water was processed with every batch of samples in the same way as the samples.

The same real-time PCR assay was performed on 200 vaginal mucus samples from the 50 heifers in weeks 4, 5, 6, 7 of exposure to infected bulls. Samples were prepared for PCR with the two heat lysis sample processing protocols that demonstrated higher sensitivity and a larger number of detected positives, as well as with the nucleic acid extraction protocol that utilized a commercial magnetic bead-based sample disruption kit^e (Table 2.1). PCR results were compared to culture results.

Real-time PCR technique

All real-time PCR tests were completed with SYBR Green real-time PCR technique as described by Chaban et al. in 2012 [7], using the VenSF/VenSR primer set. All samples were tested in duplicates. The master mix solution for each reaction included 12.5 µl of SYBR Green mix^f, 1 µl (400nM) of forward primer^g, 1 µl (400nM) of reverse primer^h, 8.5 µl of ultrapure water, and 2 µl of template DNA, in a final volume of 25 µl.

A standard curve was used in order to appreciate the efficiency of PCR reaction. The standard curve consisted of a dilution series of a plasmid containing the 142 bp Hum insert. The dilution series started with 10⁷ plasmids/reaction and sequentially decreased concentrations to end with the least concentrated plasmids, 10⁰ plasmids/reaction.

Samples were considered positive if both duplicates had a threshold cycle value and the melt curve contained a specific product peak. For melt curve analysis the melting temperature of 78.5±0.5°C was considered as specific for Cfv PCR product on the thermocyclerⁱ. Samples were considered negative if no threshold cycle was recorded or if the melt curve analysis showed a non-specific product peak for both replicates. Samples were called “suspicious” when one duplicate met the negative criteria and another tube met the positive criteria.

All suspicious samples were tested a second time by the same PCR assay, using the same sample preparation protocol. Those that remained suspicious after the second run were tested once again by the same PCR assay, using the same sample preparation protocol, and only clear positive ones were called positives; others were called negatives.

2.3.3. Statistical analyses

All data were entered into a spreadsheet and analyzed using commercial statistical software^J. Descriptive statistics and comparisons were performed.

Cohen's kappa coefficient was calculated in order to measure the agreement between the results of vaginal mucus culture based on morphology and conventional PCR of the colonies consisted in morphology with *C. fetus* [13].

To account for the correlation within samples and heifers because every heifer was sampled more than once and each sample was processed more than once, the sensitivity and specificity was analyzed with generalized estimating equations (GEE) logistic regression model in order to correct possible violations of the independence assumption. For sensitivity, culture-positive samples were selected and then a logistic regression model created with one predictor (test type). For specificity, the culture-negative samples were selected but real-time PCR results variables were coded in an opposite way to be sure to get specificity, not the false positive rate. A 95% confidence interval for sensitivity was calculated for each method. The same actions were also done for specificity.

All models were assessed for outliers and goodness of fit. Differences were accepted as statistically significant at the 95% confidence limit when $P < 0.05$ [13].

2.4. RESULTS

2.4.1. Results of culture, confirmed by multiplex conventional PCR

The results of culture of vaginal mucus from the heifers, collected in weeks 4–7 of exposure to the Cfv infected bulls (Table 2.2), demonstrated that week 6 had the largest proportion of positives (Week 4, 30%; Week 5, 24%; Week 6, 38%, Week 7, 24%). Therefore, the samples of week 6 were chosen to process with four heat lysis sample preparation protocols. In this study culture positive results based on morphology were consequently confirmed by multiplex conventional PCR assay. There was moderate agreement ($\kappa=0.48$, 95% CI: 0.48–0.49) between the results of two diagnostic methods when compared in weeks 4–7 of exposure.

2.4.2. Comparison of heat lysis sample preparation protocols

The results of real-time PCR where samples were processed with four heat lysis protocols, demonstrated that protocols III and IV were easier to perform, had a higher proportion of detected positives (Protocol I, 6%; Protocol II, 14%; Protocol III, 16%; Protocol IV, 16%) and a lower number of suspicious results (Protocol I, 16%; Protocol II, 18%; Protocol III, 6%; Protocol IV, 12%) than protocols I and II (Table 2.3a). When suspicious samples were processed again with the same protocol, there were more reduction of suspicious results, particularly in protocols I and II (Table 2.3b). The remaining suspicious samples were processed one more time and all of them were negative.

Protocol I had the lowest number of test-positive results and none of them were cultured positive (Table 2.4). The crude sensitivity of protocol IV was 1.5 times higher than that of protocol II and 1.2 times higher than that of protocol III. However, logistic regression analysis did not detect significant difference in sensitivity between protocols IV and II ($P=0.46$) and protocols IV and

III ($P=0.72$). The crude specificity of protocol I was the same as protocol II, and protocol III was the same as of protocol IV. Logistic regression analysis did not detect a significant difference in specificity between protocols ($P=0.69$).

Therefore, protocol III and IV were the best choices for further comparison to the nucleic acid extraction protocol (Protocol V) that utilized a commercial magnetic bead-based sample disruption kit^e because of higher crude sensitivity and larger number of detected positives.

2.4.3. Comparison of heat lysis sample preparation protocols to magnetic bead-based extraction method

To compare heat lysis sample preparation protocols III and IV to Protocol V (magnetic bead-based extraction), 200 samples were processed with these three methods. Protocol V demonstrated the largest proportion of detected positives (Protocol III, 19.5%; Protocol IV, 15.5%; Protocol V, 21%) and suspicious test results (Protocol III, 9.5%; Protocol IV, 15.5%; Protocol V, 20.5%) compare to culture (Table 2.5a).

All suspicious samples were tested a second time using the same protocol and the number of suspicious results reduced in all protocols, but Protocol V had the largest reduction (Table 2.5b). The remaining suspicious samples were processed one more time and all of them tested negative (Table 2.6).

Logistic regression GEE model analysis demonstrated that the sensitivity of Protocol V (48.4%) was significantly higher than protocol IV (29.4%) ($P=0.02$) and tended to be higher than Protocol III (35%) ($P=0.16$). However, Protocol V had significantly lower specificity (78.9%) than Protocol IV (88.2%) ($P=0.01$), but there was no significant difference in specificity between Protocol V (78.9%) and Protocol III (81.1%) ($P=0.62$).

2.5. DISCUSSION

In this study the results of Cfv real-time PCR were compared for bovine vaginal mucus samples, prepared using heat lysis and magnetic bead based sample preparation techniques, to results of direct, selective culture of the same samples. The sample processing method, designed and successfully applied for bull preputial samples [7], demonstrated relatively low sensitivity compared to culture, confirmed by multiplex conventional PCR, when tested on vaginal mucus samples in this study. All modifications of that heat lysis protocol demonstrated low sensitivity when were compared to culture, confirmed by conventional PCR; however, the most satisfactory results were achieved with protocols involving fewer processing steps. The magnetic bead-based extraction method tended to be the comparatively more sensitive than heat lysis methods but required too much time and resources to be practical for routine use.

The preputial samples mostly consisted of smegma and blood; therefore, the DNA isolation protocol designed for preputial samples contained several steps with a purpose to eliminate possible inhibitors [7]. Bovine CVM comprises two phases: aqueous and gel [14]. The aqueous phase contains low molecular weight compounds that are dissolved in up to 95% water [10]; the gel phase is formed mainly by insoluble high molecular weight glycoproteins (mucins) [14, 15]. These highly glycosylated proteins are probably the main factor, responsible for the rheological properties of CVM [15]. Biophysical and biochemical properties of CVM are controlled by sex steroids during the estrus cycle [14]. For instance, during the pre-ovulatory period CVM becomes less viscous and more hydrated, facilitating the posterior movement of spermatozoa during breeding [15], while in the luteal phase hydration decreases and viscosity increases, preventing spermatozoa migration [15–17].

CVM is secreted during the whole cycle, but its volume increases at estrus [17]. Noonan et al. noticed an inverse relationship between the dry matter content in CVM and the degree of crystallization [16]. Decreasing in proportion of salts such as NaCl, KCl and CaCl₂ in the aqueous part of CVM leads to decreasing water content. Such changes cause a decrease in crystallization and a relative increase in other salt components in the dry residue. During the estrous cycle, the dry matter concentration in CVM reaches a minimum value on the day of estrus and a maximum value at the mid-cycle and increased from the day of estrus to the 19th day of gestation, while the extent of crystallization decreased. Crystallization occurred to the greatest extent on the day of estrus [17, 18]. Therefore, whenever CVM is collected it appears “gelly” due to either high viscosity or crystallization.

We speculate that the biophysical properties of vaginal mucus may have caused DNA loss during the isolation process. Dilution steps in the heat-lysis protocol that were designed to reduce PCR inhibitors may have resulted in lost DNA. The gelatinous consistency of the mucus also made it difficult to accurately pipette and may have impacted the distribution of the organism and DNA in the sample. It seemed impossible to homogenate these samples prior to pipetting. Even though no quantitative analysis was performed, it can be appreciated from this study that heat lysis protocols, where pellet or supernatant were not discarded, had greater sensitivity.

Different DNA isolation protocols can be utilized on clinical samples, collected from farms with a history of infertility. In one study [18], 94 CVM samples, collected mainly during the estrous cycle, were incubated in a transport enrichment medium (TEM) at 37°C for 5 days, and then processed with cetyltrimethylammonium bromide (CTAB) for DNA extraction for consequent Hum’s primer set-based PCR [5]. According to the authors the 5-day period of pre-enrichment in TEM provided a multiplication of the bacteria and increase in PCR detection and the CTAB

extraction protocol resulted in Cfv detection in 34% of samples [18]. In the same study, from 277 prepuce samples, CVM and abomasum contents of aborted fetuses, 24% were tested positive for *C. fetus* using PCR, while only 2.8% were positive by bacterial isolation in culture [18]. This method required five days to perform and the large number of detected positives was most likely due to maintaining viability of the pathogen. The objective of our research, however, was to test rapid sample processing protocol for bovine CVM that can be used under field conditions when viability of Cfv could be compromised.

The most satisfactory results were achieved with modified heat lysis protocols involving fewer processing steps. The two most sensitive protocols were compared to the magnetic bead-based extraction method. The magnetic bead-based nucleic acid isolation kit, used in this study, was designed for rapid high throughput purification of DNA and RNA for use in PCR applications. According to the manufacturer^e it can be utilized for different types of biological and environmental samples.

All sample preparation methods for bovine vaginal mucus that were compared in this study had a low sensitivity and high specificity relatively to culture, which remains the gold standard according to the World Organization for Animal Health release of 2012 (19). More positives were detected by culture and confirmed by conventional PCR than by real-time PCR. In our study, culture was performed under ideal conditions: plating within four hours, and keeping reasonable environmental temperatures prior to plating. This allowed maintaining viability of Cfv, which is the biggest challenge under field conditions. We then applied conventional PCR directly on the suspect colonies which increased the specificity of the culture. The agreement (kappa) between culture and conventional PCR was moderate in this study since many samples

were culture positive based on morphology but were negative accordingly to conventional PCR results.

Even though the magnetic bead-based extraction method tended to be the most sensitive, it required much more time and resources to perform than the other two. The entire isolation procedure for bovine vaginal mucus with the commercial magnetic bead-based sample disruption kit required about 3 hours to perform. Protocol III was the easiest to perform and there was no statistically significant difference between Protocol III and V in sensitivity and specificity. The sample preparation method for PCR, developed for preputial samples, can be potentially utilized for vaginal mucus when all dilution steps are skipped.

Our study has been performed on samples that were frozen and thawed several times since every sample was processed with different methods. Initially, PCR results of 50 samples, processed with four heat lysis protocols, were compared to the culture. Then, all 200 samples were processed with protocols III, IV and V. Protocols III and IV were performed at the same time. Protocol V was performed last.

Rapid freezing results in intracellular ice crystal formation, causing membranes to rupture, whereas slow cooling allows water to leach out reducing crystal formation, but leads to cell rupture due to osmotic pressure imbalance. Ice crystal-induced damage to organelle structures leads to activation of rescue systems, associated with energy generation that results in production of free radicals and oxidative stress to DNA [20]. Intracellular (DMSO, glycerol, and ethylene glycol) or extracellular (sucrose, dextrose, and polyvinylpyrrolidone) cryoprotectants can be added to samples. Intracellular agents prevent the formation of ice crystals and extracellular

agents reduce the hyperosmotic effect during freezing [20]. Therefore, further testing of the DNA isolation methods should be performed utilizing cryoprotectants or on fresh samples.

2.6. CONCLUSIONS

The heat lysis sample preparation protocol, previously utilized for bovine preputial samples, required some modifications in order to adapt it to CVM. Release of DNA directly from a CVM sample by a modified heat lysis protocol (Protocol III) was easy to perform and gave a sensitivity of 35% and specificity of 81% in subsequent real-time PCR. A magnetic bead-based nucleic acid extraction protocol demonstrated sensitivity of 48%; however, this protocol is time and resource consuming. Finally, these two methods should be further compared on fresh CVM samples in order to remove possible bias of freezing-thawing.

^aContinental Plastics, Vetsource Canada, Cambridge, ON; Item #E6-5415

^bContinental Plastics, Vetsource Canada, Cambridge, ON; Item #B6-4775

^cMillipore, Billerica, MA, USA

^dGasPak™ EZ Campy Pouch™ System, BD diagnostics, Mississauga, ON, Canada

^eMagMAX™ Total Nucleic Acid Isolation Kit (Bead-Based Sample Disruption), Applied Biosystems, Ambion; Part Number AM1840

^fIQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA, 94547

^gVenSF: 5'-CTT AGC AGT TTG CGA TAT TGC CAT T-3' [11]

^hVenSR: 5'-GCT TTT GAG ATA ACA ATA AGA GCT T-3' [11]

ⁱBio-Rad MyiQ, Bio-Rad Laboratories, Inc.

^jStata 12 IC, Stata Corp., College Station, TX

Table 2.1. Sample processing protocols for vaginal mucus real-time PCR used in this study

Protocol	Description
I	1) 200 µl of sample was centrifuged in a microfuge tube for 5 min at $12\,000 \times g$ 2) supernatant discarded 3) 100 µl of ultrapure H ₂ O was added directly on top of the pellet 4) vortexed for ~5 seconds 5) heated at 95°C in a water bath for 10 min 6) centrifuged 30 seconds at $2\,000 \times g$ 7) 20 µl of supernatant was added to 180 µl of ultrapure H ₂ O (1:10 dilution) [7].
II	Same as protocol I, but step 7 was skipped
III	1) 200 µl of sample was heated at 95°C in a water bath for 10 min 2) centrifuged 30 seconds at $2\,000 \times g$
IV	1) 200 µl of sample was heated at 95°C in a water bath for 10 min 2) centrifuged 30 seconds at $2\,000 \times g$ 3) diluted 1:10
V	Samples were processed according to the manual for the commercial magnetic bead-based nucleic acid isolation kit ^e

Table 2.2. Results of vaginal mucus samples culture, confirmed by multiplex conventional PCR, from the 50 heifers, naturally exposed to Cfv infected bulls

Culture results	Weeks of natural exposure (number of samples)				Total
	4	5	6	7	
Positive	15	12	19	12	58
Negative	35	38	31	38	142
Total	50	50	50	50	200

Table 2.3a. Comparison of Cfv real-time PCR results for bovine vaginal mucus samples, prepared with heat lysis protocols (week 6 samples), to results of culture, confirmed by multiplex conventional PCR, n=50

Culture results	Sample processing protocols results (number of samples)															
	I				II				III				IV			
	P*	S**	N***	Σ	P	S	N	Σ	P	S	N	Σ	P	S	N	Σ
P	0	5	14	19	4	3	12	19	4	3	12	19	5	3	11	19
N	3	3	25	31	3	6	22	31	4	0	27	31	3	3	25	31
Σ	3	8	39	50	7	9	34	50	8	3	39	50	8	6	36	50

*P-positive; ** S-suspicious; *** N-negative

Table 2.3b. Comparison of Cfv real-time PCR results for bovine vaginal mucus samples, prepared with heat lysis protocols (week 6 samples), to results of culture, confirmed by multiplex conventional PCR, after re-running of suspicious samples, n=50

Culture results	Sample processing protocols results (number of samples)															
	I				II				III				IV			
	P*	S**	N***	Σ	P	S	N	Σ	P	S	N	Σ	P	S	N	Σ
P	0	2	17	19	4	0	15	19	5	2	12	19	5	3	11	19
N	3	0	28	31	3	1	27	31	4	0	27	31	3	3	25	31
Σ	3	2	45	50	7	1	42	50	9	2	39	50	8	6	36	50

*P-positive; ** S-suspicious; *** N-negative

Table 2.4. Comparison of Cfv real-time PCR results for bovine vaginal mucus samples, prepared with heat lysis protocols, to results of culture, confirmed by multiplex conventional PCR, after called suspicious samples negative, n=50

Protocol, result		Culture, result			Sensitivity / Specificity
		Positive	Negative	Σ	
I	Positive	0	3	3	Sens=n/a Spec=90.3% (95% CI: 73.9 –96.8%)
	Negative	19	28	47	
	Σ	19	31	50	
II	Positive	4	3	7	Sens=21.0% (95% CI: 8.1–44.5%) Spec=90.3% (95% CI: 73.9 –96.8%)
	Negative	15	28	43	
	Σ	19	31	50	
III	Positive	5	4	9	Sens=26.3% (95% CI: 11.4–49.8%) Spec=87.1% (95% CI: 70.0–95.1%)
	Negative	14	27	41	
	Σ	19	31	50	
IV	Positive	6	4	10	Sens=31.6% (95% CI: 14.9–54.8%) Spec=87.1% (95% CI: 70.0–95.1%)
	Negative	13	27	40	
	Σ	19	31	50	

Table 2.5a. Comparison of Cfv real-time PCR results for bovine vaginal mucus samples, prepared with different protocols, to results of culture, confirmed by multiplex conventional PCR, n=200

Culture results	Sample processing protocols results (number of samples)											
	III				IV				MagMAX			
	P*	S**	N***	Σ	P	S	N	Σ	P	S	N	Σ
P	17	7	34	58	16	8	34	58	21	16	21	58
N	22	12	108	142	15	23	104	142	21	25	96	142
Σ	39	19	142	200	31	31	138	200	42	41	117	200

*P-positive; **S-suspicious; ***N-negative

Table 2.5b. Comparison of Cfv real-time PCR results for bovine vaginal mucus samples, prepared with different protocols, to results of culture, confirmed by multiplex conventional PCR, after the re-running of suspicious samples, n=200

Culture results	Sample processing protocols results (number of samples)											
	III				IV				V			
	P*	S**	N***	Σ	P	S	N	Σ	P	S	N	Σ
P	20	2	36	58	17	1	40	58	28	3	27	58
N	26	5	111	142	16	2	124	142	29	2	111	142
Σ	46	7	147	200	33	3	164	200	57	5	138	200

*P-positive; **S-suspicious; ***N-negative

Table 2.6. Comparison of Cfv real-time PCR results for bovine vaginal mucus samples, prepared with different protocols, to results of culture, confirmed by multiplex conventional PCR, after called suspicious samples negative, n=200

Protocols, results		Culture, results			Sensitivity / Specificity
		Positive	Negative	Σ	
III	Positive	20	26	46	Sens=35.0% (95% CI: 24.2%–48.0%) Spec=81.1% (95% CI: 72.7%–87.3%)
	Negative	38	116	154	
	Σ	58	142	200	
IV	Positive	17	16	33	Sens=29.4% (95% CI: 19.6%–41.6%) Spec=88.2% (95% CI: 82.1%–92.4%)
	Negative	41	126	167	
	Σ	58	142	200	
V	Positive	28	29	57	Sens=48.4% (95% CI: 36.9%–60.1%) Spec=78.9% (95% CI: 71.4%–84.8%)
	Negative	30	113	143	
	Σ	58	142	200	

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3. EFFICACY OF A COMMERCIAL VACCINE FOR BOVINE GENITAL CAMPYLOBACTERIOSIS IN PREVENTING INFECTION AND REPRODUCTIVE LOSS IN CATTLE

In chapter 2 Cfv real-time PCR results for samples, prepared using heat lysis and magnetic bead based sample preparation techniques, were compared to results of direct, selective culture of bovine vaginal mucus. The heat lysis sample preparation protocol, previously utilized for bovine preputial samples (Chaban et al. 2012. Can J of Vet Res; 76:166), did not demonstrate satisfactory performance for vaginal mucus and required some modifications. Modified heat lysis protocols were compared to culture, confirmed by multiplex conventional PCR, and to results of real-time PCR samples, prepared with a commercial magnetic bead-based extraction kit. Release of DNA directly from a CVM sample by a modified heat lysis protocol was easy to perform but demonstrated low sensitivity and could be performed as a rapid screening approach for BGC after validating on fresh samples. The magnetic bead-based extraction protocol demonstrated slightly higher sensitivity but was time and resource consuming to perform. The biggest number of positive samples was detected with selective culture of bovine vaginal mucus, when colonies, consistent with C. fetus morphology, were subjected to conventional PCR using MG3F/MG4R and VenSF/VenSR primers. This method was chosen for testing vaginal mucus samples in the vaccine trial, described in chapter 3.

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3.1. ABSTRACT

Introduction — Bovine Genital Campylobacteriosis occurs worldwide causing early pregnancy loss and temporary infertility. While commercial vaccines are available, their effectiveness has not been well documented in the peer-reviewed literature.

Objective — To compare the risk of infection and reproductive failure in heifers, exposed to BGC through natural breeding, that were vaccinated with a multivalent commercial vaccine containing *C. fetus* antigen, to heifers vaccinated with a comparable product without *C. fetus*.

Study design — Prospective randomized controlled clinical trial

Animals — 50 cross-bred beef heifers

Materials and methods — Heifers were randomized to treatment and control groups and then exposed to bulls, infected with BGC. The animals in the control group were vaccinated with a commercial vaccine against Infectious Bovine Rhinotracheitis virus, BVD, Parainfluenza-3, Respiratory Syncytial Virus, and *Leptospira interrogans*. The treatment group was vaccinated with a similar product also containing the *C. fetus* antigen. Vaginal mucus samples were collected and cultured for 16 weeks; tiny translucent colonies were Gram-stained and if consistent with *C. fetus* morphology were subjected to conventional PCR using MG3F/MG4R primers targeting carbon starvation gene protein in both *C. fetus* subspecies and VenSF/VenSR primers targeting *parA* gene fragment in *C. fetus* spp. *venerealis*. Pregnancy status was determined by trans-rectal ultrasonography and concentration of pregnancy-specific protein B in serum. Serum neutralizing antibody titers to *C. fetus* were also measured.

Results —There was no significant difference between groups either in risk of isolating CfV ($P>0.17$) or in the proportion of heifers that cultured positive at least once ($P=0.42$). Similarly, there was no difference in the median number of times that heifers cultured positive for CfV from vaginal mucus samples ($P=0.24$) and the rate at which heifers from control and treatment groups first cultured positive ($P=0.67$). The proportion of heifers that were pregnant each week during the study did not differ by treatment group, based on either ultrasound ($P>0.31$) or serum PSPB ($P>0.31$). The time to first pregnancy detected by ultrasonography did not differ between treatment and control groups for heifers ever diagnosed as pregnant ($P=0.30$) and those that remained pregnant at the end of the study ($P=0.70$). There was no significant difference in the proportion of animals, ever diagnosed pregnant during the study, by treatment group (Tx=48%, C=36%; $P=0.39$) and in pregnancy loss rates (Tx=75.0%, C=67%; $P=0.28$). However, heifers that aborted during the study were 4 times more likely to be culture positive for BGC than those that did not abort ($P=0.01$). Heifers in the treatment group that were not pregnant at the end of the study cultured positive 1.5 times more often than pregnant animals ($P=0.04$); nonpregnant heifers from control group were 4 times more likely to culture positive ($P=0.01$). There was no difference in the number times cultured positive in heifers that were not pregnant at the end of the study ($P=0.14$). Neither the maximum detected MAT antibody titers ($P=0.57$) to *C. fetus* nor the proportion of positive MAT titers ($P=0.99$) differed by treatment. However, the mean ELISA antibody to *C. fetus* concentrations after vaccination were more than 2 times higher in the treatment group than in the control group ($P<0.02$).

Conclusions — Vaccinating heifers with a multivalent commercial vaccine, containing *C. fetus* antigen, according to the label directions did not significantly reduce infection rates or improve pregnancy when challenged with exposure to bulls, infected with BGC.

3.2. INTRODUCTION

Bovine Genital Campylobacteriosis (BGC) is a venereal disease characterized by early pregnancy loss and temporary infertility [1,2]. It is caused by the bacteria *Campylobacter fetus* subspecies *venerealis* (Cfv). BGC occurs worldwide, wherever natural service is used for breeding [3–8]. Pregnancy loss is often not detected until pregnancy examination or a high number of females are noticed exhibiting estrus after the breeding season [9]. Economic losses associated with acute outbreaks of Cfv in susceptible herds and chronic infections can be severe [1,4]. BGC is maintained and transmitted by carrier bulls [9]. Infection of a susceptible cow or heifer during breeding results in endometrial inflammatory changes and potentially in death and resorption of the conceptus [1,10].

The advent and use of artificial insemination, testing and vaccination has decreased the occurrence of BGC in many countries [8,10]. However, the beef industry, unlike dairy business, primarily uses natural breeding [11, 12]. Systemic immunization could prevent or eliminate Cfv infection by inducing IgG antibodies in genital secretions and serum, shortening infection and reducing reproductive losses. Vaccine-induced immunity has been reported to be effective but short-lived and advised that vaccination be boosted bi-annually to ensure adequate herd protection [11].

The relatively small number of peer-reviewed reports to date evaluating the effectiveness of vaccination of bulls [11, 13–17, 18, 19], cows and heifers [16, 19–25, 26–28] were summarized in the Table 3.1. Both monovalent [11, 13–21, 23, 25–28] and polyvalent [22, 24] vaccines have been used for vaccination against BGC in heifers. Only a small number of studies have reported commercial vaccines [11, 16, 18, 21, 22, 24, 26]. The remainders were prepared from local

strains [13–16, 20, 22–25, 27, 28] utilizing oil [13, 15, 16, 20, 23, 24, 27, 28] or aluminum compounds adjuvants [22]. Incomplete Freund's adjuvants have also been used [14, 25]. Usually systemic vaccination for BGC was described in vaccine trials [11, 13–17, 19–25, 26–28], except in one study mucosal vaccination was performed as an addition to systemic vaccination [24]. Animals were challenged by natural exposure to infected animals of opposite sex [14–16, 19, 20, 22, 26], intra-vaginal [21, 23, 25] or intra-preputial [11] Cfvi culture infusion, or both natural exposure and culture infusion [17, 24, 27, 28].

Vaccination for BGC with monovalent vaccine has been reported as effective method of preventing infection and associated reproductive losses; however, most of the studies are more than 30 years old, performed on experimental [13, 14, 19, 20, 23, 25, 27, 28] and commercial vaccines [11, 17, 19, 21, 26]. Nearly half of early published studies about monovalent experimental vaccines were neither contained information about randomization of animals to treatment groups nor statistically conclusive [13, 14, 16, 23, 25]. For instance, vaccination of 188 bulls with vaccine, prepared with local antigen, resulted in their non-infected status after natural exposure in infected areas for 1 year [14]. Another trial reported successful vaccination of 46 heifers and 4 bulls [16]. However, there was no information found in these papers about the method how treatment groups were formed and only percentages were reported in these trials without appropriate data analysis.

Studies of monovalent commercial vaccines in most cases were randomized [11, 17, 18, 26] and reported statistically analyzed data [17, 18, 21, 26]. Study of monovalent commercial vaccine, prepared from an Australian isolate of *Campylobacter fetus* subspecies *venerealis* biovar *intermedius* (Cfvi), demonstrated 7 times less infection in the group of vaccinated heifers, compare to non-vaccinated [21]. This study reported statistically significant data, but nothing

was mentioned how treatment and control groups were formed. Randomized and statistically conclusive study of monovalent commercial vaccine was performed on 12 infected bulls and reported increasing in serum antibodies to *C. fetus* titers in vaccinated bulls, and when exposed afterwards to virgin heifers resulted in non-infection status of bulls and heifers [17].

There have been reports of utilizing monovalent vaccine with a purpose to cure BGC infection [14, 15, 23, 25]. One study suggested that active systemic immunization would be able to eliminate vaginal carrier state in heifers [25]. Later, Cfv bacterin in oil adjuvant was tested, but recommended as an additional strategy only [15].

The effectiveness of commercial polyvalent vaccines has not been as clearly described. There have been two peer-reviewed, recent studies [22, 24] that were well designed clinical trials with appropriate data analyses. g. In one study, polyvalent commercial vaccine, formulated with inactivated antigens of *Leptospira interrogans* serovar pomona, *Haemophilus somnus*, *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus* and viruses of the infectious bovine rinotracheitis and bovine viral diarrhoea in aluminium hydroxide adjuvant, was used to vaccinate heifers that were consequently naturally challenged for 60 days [22]. Vaccinated and control heifers had poor reproductive performance and did not demonstrate significant systemic antibody level, detected by immunofluorescent antibody test (IFAT) [22].

When commercial and experimental dual vaccines containing *Tritrichomonas foetus* and *Campylobacter fetus* antigens were tested, vaccinated heifers resisted or quickly cleared both pathogens, had a higher pregnancy rate, and a higher systemic immune response. The experimental vaccine, containing antigens from local strains of *C. fetus* and *T. foetus*, given both subcutaneously and intravaginally, was superior to the commercial vaccine [24]. Whether that

was due to the fact of using local strains of the pathogens in experimental vaccine or due to local application of that preparation remained questionable.

It could be suggested from the literature review that monovalent vaccines demonstrated better effectiveness than polyvalent; however, most studies on monovalent vaccines were done quite long time ago and about half of these trials either contained no information regarding randomization [15, 21] or data that was not analyzed appropriately [11], or both [13, 14, 16, 23, 25]. The majority of such studies were performed on vaccines, prepared from the antigens of local strains of *C. fetus* [13, 15, 16, 20, 23, 24, 25], sometimes combining systemic and local routes of vaccination [24], which could be another reason of their improved effectiveness. There are a few reports purportedly demonstrating successful use of commercial monovalent vaccines [11, 17, 21]. However, trials with commercial monovalent vaccines were performed on limited number of animals (12–33) resulting in a small sample size [11, 17, 18, 21, 26], while the number range of animals used in the studies with experimental monovalent vaccines was 4–321 [13, 15, 16, 20, 23, 24, 25]. However, studies of commercial vaccines in most cases were randomized and statistically conclusive [18, 19, 26].

Currently, commercial vaccines are more convenient and less expensive to use for producers than those, exclusively prepared for every single farm or area, based on local strains of pathogens (autogenous). Preparation of autogenous vaccines is not really practical, especially when considering withdrawal times and food safety. When comparing monovalent and polyvalent vaccines, the latter are preferable to use in modern farms and ranches, since one vaccination against many pathogen is less stressful for animals and personnel than multiple injections. There was only one peer-reviewed study in the last decade [22] concerning polyvalent vaccine for BGC. This vaccine contained *Leptospira interrogans* serovar pomona,

Haemophilus somnus, Cff and viruses of infectious bovine rinotracheitis and bovine viral diarrhoea in addition to Cfv. Since a small number of animals were used in that trial, the efficacy of such vaccination as reported was questionable [22]. Therefore, there is a need to better understand the efficacy of commercially available Cfv vaccines so that veterinarians can be informed and make effective decisions on vaccination practices for herds. Well-designed clinical trials with commercially available polyvalent vaccine containing *C. fetus* antigen, followed by data analysis with updated statistical techniques would be appropriate.

The overall purpose of this project was to describe the differences in infection rate and reproductive performance between two groups of heifers naturally exposed to Cfv-infected bulls. The first group was vaccinated with polyvalent vaccine containing *C. fetus* antigen and the second was vaccinated with similar polyvalent vaccine that did not contain *C. fetus* antigen. The primary objectives were to compare the percentage of heifers that became infected with Cfv, the proportion of heifers that became pregnant, and the difference in days to pregnancy between the two groups. A secondary objective was to describe and compare serum concentrations of *C.fetus* antibodies between the two treatment groups.

3.3. MATERIALS AND METHODS

3.3.1. Animals and treatments

All animal procedures were performed in accordance with the Canadian Council of Animal Care and approved by the University of Saskatchewan Animal Research Ethics Board (Protocols #20100061 and #20100077). A sample size of 25 heifers per treatment group was calculated based upon an expected pregnancy proportion of 30% and 75% in the control and treatment groups, respectively, with 80% power and 95% confidence. [29].

Fifty cross-bred beef heifers were housed during May and June 2013 in a 35m x 35m drylot pen at the university research farm^a. The heifers were fed a diet of mixed hay, barley silage and mineral supplement according to NRC requirements. Each heifer was examined trans-rectally via ultrasonography using a commercial scanning device^b with 5.0 MHz 10 cm linear probe for the presence of a corpus luteum as evidence of cyclicity (Weeks -4 and 0) (Table 3.1). Heifers were paired by body weight into 25 replicates. Animals within each replicate were then randomized by coin toss to 1 of 2 vaccination treatments where “heads” would mean BGC vaccination and “tails” would mean control vaccination.

The control group (C) was vaccinated with 2 doses (2 ml/dose) of a commercial vaccine^c, containing the following antigens: Bovine Rhinotracheitis, Bovine Virus Diarrhea, Parainfluenza-3, Respiratory Syncytial Virus, *Leptospira interrogans* (serovars canicola, grippotyphosa, hardjo, icterohaemorrhagiae, pomona) with a proprietary adjuvant, subcutaneously 7 and 4 weeks before breeding. The treatment group (Tx) got 2 doses (2 ml/dose) of the commercial vaccine^d, containing the same components as for control plus the *Campylobacter fetus* antigen, subcutaneously 7 and 4 weeks before breeding.

Heifers were exposed to 2 of 3 Cfv-infected Black Angus bulls that were genetically distinct from the heifers for a period of 63 days (July 07–September 02, 2013). While heifers were only exposed to two of the bulls at a time, a third bull was kept as spare and used in case of injury or disease. The bulls were vaccinated with the same vaccine^c as heifers from the control group and with the vaccine^e for *Fusobacterium necrophorum*. During the breeding period, the heifers and bulls were rotationally grazed on four 12-acre pastures.

A chin-ball marker^f was placed on each of the bulls to monitor the heifer's exposure to the bulls. A different paint color was used for each bull. Heatmount detectors^g were also placed on the tail head of each heifer at the beginning of the trial (day 0) to identify heifers in estrus. The Heatmount detector and chin-ball paint were monitored visually and recorded once daily (for 30–60 minutes each morning) during the first 3 weeks of the trial to monitor bull exposure.

3.3.2. Data and sample collection

The heifers and bulls were walked into corrals, adjacent to the pastures, and then moved through a handling facility to facilitate sample collection. During the procedures, animals were restrained in a manual squeeze chute and haltered for blood collection. A summary of the vaccination, data and sample collection schedule is provided in Table 3.2.

Preputial scrape sampling

Preputial scrape samples were collected from the 3 bulls weekly from week 0 to 10 for culture and real-time PCR to monitor their Cfv infection status. Bulls were sampled using a previously described aspiration technique [30]. Cryovials with samples were transported back to the lab in a styrofoam box with warm water bags within 2h of collection, maintaining environmental temperature at approximately 25°C.

Vaginal mucus sampling

Two vaginal mucus samples were collected before bull exposure to ensure that the heifers were free from Cfv infection before beginning the trial. Heifers were then sampled weekly from weeks 1 to 10 of exposure. Mucus samples were also collected at weeks 12, 14 and 16 (Table 3.2).

Sampling was performed by aspiration using a 25'' pipette^h, connected to a 20-ml syringe. Prior to aspiration, approximately 2 ml of phosphate buffered saline (PBS) was passed through the pipette into the syringe. The pipette was then placed inside a solid 12-inch plastic AI sheath protectorⁱ. The tail of each heifer was held and perineum washed with soapy warm water. The labia were then opened and the pipette inserted into the vagina while protected in the AI sheath. The pipette was then advanced through the AI sheath. The PBS in the syringe was then expelled and 15 mL of suction applied. The syringe was then maneuvered cranial and caudal moving the pipette along the vaginal walls approximately 10 times while maintaining suction. Finally, the pipette was pulled caudally into the AI sheath before removing it from the vagina to minimize fecal contamination. Once the aspiration of mucus was complete, the pipette was withdrawn and rinsed into a 4-ml cryovial tube containing 2 ml of PBS. Cryovials were transported back to the lab in a styrofoam box with warm water bags within 2h of collection, maintaining environmental temperature at approximately 25°C.

Blood sample collection

Two 10-ml blood samples were collected from the jugular vein into red-topped vacuum tubes^j on weeks -7, 0, 2 through 8, 10 and 16. The blood samples were kept at room temperature until transported back to laboratory. In laboratory the blood was centrifuged at 1,500 g for 15 minutes. The serum was then aliquoted into three 2.5 mL cryovials and then frozen at -20°C until samples collection was complete.

Trans-rectal ultrasonography for pregnancy

The heifers were examined by trans-rectal ultrasonography every Monday from weeks 4 to 16 of the study. Pregnancies were approximately aged via ultrasonography by taking into account the

size of the vesicle, fetus and cotyledons. The heartbeat of the embryo was assessed to confirm viability.

Breeding soundness examination

Each of the 3 bulls had a breeding soundness exam on weeks -4 and 11 where they were assessed for physical soundness, scrotal circumference, sperm motility and morphology according to Barth (2013) [31].

3.3.3. Laboratory testing:

Culture of vaginal mucus and preputial samples, confirmed by multiplex conventional PCR

Upon arrival at the lab, an aliquot of 300 µl was taken from the cryovial and spread onto a 0.65-µm mixed cellulose ester membrane filter^k, placed on 5% blood agar plates and incubated at 37°C under microaerobic conditions with an integrated chemical packs^l for rapid carbon dioxide (CO₂) generation [32]. Plates were examined after 72h of incubation and colonies, consistent with *Campylobacter fetus* morphology, were Gram stained. Plates that contained no evidence of *Campylobacter* colonies were incubated for an additional 72h. Colonies were not counted, but those with Gram stain results, consistent in morphology with *Campylobacter*, were subjected to a conventional multiplex PCR [33], using the primers MG3F/MG4R and VenSF/VenSR, as previously described [30, 32, 33], to determine the species and subspecies.

Pregnancy-specific protein B (PSPB)

The concentration of pregnancy-specific protein B (PSPB) in the serum was measured using a commercial kit^m at a commercial laboratoryⁿ. Sera were frozen and tested together at one time at

the end of the trial to minimize variation. The testing was performed utilizing the “sandwich” enzyme-linked immunosorbent assay (ELISA) [34]. Accordingly to the kit manual^m the most accurate results are received when the test is performed after 28 days post-breeding, giving greater than 99 percent of accuracy when an animal is categorized as open.

Standards of known PSPB concentrations were run with each assay to determine the optical density (OD) values for assigning pregnant/not pregnant ranges, the “cut off point” was 0.15 (Table 3.3).

Antibodies to *C. fetus*

Serum neutralizing antibody titers to *C. fetus* were measured in this study with microscopic agglutination test (MAT) and ELISA by a commercial laboratory^p. ELISA was performed as previously described for quantification of IgG antibodies against *C. fetus* after vaccination [35].

For the MAT test sera were twofold serially diluted in U-bottom microtiter plates (50 µl per well). The diluent was phosphate buffered saline (PBS) containing 0.1% gelatin. Fifty µl of *C. fetus* inactivated antigen (0.2% formalized, 54–58 %T at 550 nm) was added to each well and the plates were incubated at 37°C for 16–24 hours. The plates were held an additional 16–24 hours at 15–30°C before final reading.

A positive reaction in MAT was defined in case when a smooth mat of cells covered the entire bottom of the well with no defined button of un-reacted antigen. A negative reaction ranged from a partial smooth mat to a compact button of unreacted antigen. The titer was expressed as the highest serum dilution causing a positive reaction. MAT titers 64 and above were considered as positive.

3.3.4. Data analyses

All data were entered into a spreadsheet before analysis, and appropriate descriptive statistics were reported for each variable, using commercial statistical software^P. The 95% confidence intervals (95% CI) were reported for means and proportions, while inter-quartile ranges (IQR) were reported for medians.

Heat detection and bull exposure

The proportions of heifers in estrus and mounted by bulls were compared between treatment groups, using Fisher's exact test, during the periods when these events happened the most. The results were reported as odd ratios (OR) with 95% confidence intervals (95% CI) [29, 36]. The median numbers of days from the start of the study to the first estrus and to the first mounting by bulls were also compared across treatment groups using Wilcoxon rank sum test [29].

Generalized Estimating Equations (GEE) with a logit link function, binomial distribution and AR(1) correlation was used to compare differences in the probability a heifer was exposed to a bull among the 2 bulls used in the study and the 2 treatment groups for the first 12 days and again for days 14 to 21, while accounting for repeated measures from individual heifers. Firstly, treatment groups, bulls, day number were used as independent variables, followed by post-estimation as margins of bulls variables; descriptive statistics and comparison of heifers exposure to different bulls were reported. Secondly, treatment groups, bulls, day number, and treatment/bull interaction were used as independent variables, followed by post-estimation as margins and pair-wise comparison of the interaction term that were reported as descriptive statistics and comparisons between exposure of every bull to different treatment groups of heifers [37]. The results were reported as OR with 95% CI [29].

Culture of vaginal samples, confirmed by multiplex conventional PCR

A GEE model with a logit link function, binomial distribution and AR(1) correlation was used to compare the proportion of vaginal mucus samples, cultured positive weekly over the period of the study, between treatment and control heifers by treatment, time, and the interaction of these two terms while accounting for repeated measures from individual heifers. Pair-wise comparison between treatment groups by week of study were reported for the interaction terms [37]. The results were reported as OR with 95% CI [29].

The proportion of heifers that had at least 1 positive vaginal mucus culture was compared by treatment and by exposure of heifers to different bulls using Fisher's exact test. The results were reported as OR with 95% CI [29].

The median number of times that heifers cultured positive during the study was compared by treatment with Wilcoxon rank sum test. The median number of times that heifers cultured positive at least once was compared between heifers, mounted by three different bulls, using Kruskal-Wallis one-way non-parametric analysis of variance (ANOVA). The time from the start of exposure to when first cultured positive for Cfv was compared by treatment with Wilcoxon rank sum test [29].

Pregnancy diagnosis by serum PSPB and ultrasonography

Cohen's kappa coefficient was calculated in order to measure the agreement between the pregnancy results, obtained by serum PSPB and ultrasonography [29]. Abortion events were estimated looking at the spreadsheet with weekly pregnancy detection data.

GEE models, using a logit link function, binomial distribution and AR(1) correlation, were used to compare the proportion of heifers, detected pregnant each week by ultrasound and then PSPB, between treatment groups in a model including study week and the interaction between treatment group and week while accounting for repeated measures from individual heifers. Pair-wise comparisons were reported between treatment groups by week of study [37]. The results were reported as OR with 95% CI [29].

Using the estimated fetal age from ultrasonography, the days from initial bull exposure to pregnancy was estimated for all heifers, diagnosed pregnant at least once during the study, and also for animals that remained pregnant at the end of the trial. Only days to initial pregnancy diagnosis was included in this analysis. That is, if heifers were pregnant, aborted and then became pregnant again, we only considered the first pregnancy.

Fisher's exact test was used to compare the risk between treatment groups that heifers were diagnosed pregnant at least once during the study, pregnant at the end of the study, aborted, and that either heifers that aborted or heifers that had not aborted were cultured positive at least once during the study [29]. The results were reported as OR with 95% CI [29].

Wilcoxon rank sum test was then fit to compare the median number days from the start of exposure to first pregnancy between treatment groups for all pregnant during the study heifers, those that remained pregnant at the end of the trial, and pregnant heifers that never aborted, as well as the heifers that aborted during the study and those that did not abort in the number of times cultured positive. Differences in the number of times cultured positive were also compared with Wilcoxon rank sum test between pregnant and non-pregnant heifers at the end of the study overall and by treatment group [29].

C. fetus antibody titer serology

Fisher's exact test was used to compare the proportion of positive titers, detected by MAT, during the study by treatment groups. The results were reported as OR with 95% CI [29]. The number of Cfv positive titers for each heifer and the maximum serum titre for *C. fetus* was compared between vaccine groups, using a Wilcoxon rank sum test [29].

A GEE model of log base 10 transformed ELISA results assuming a normal distribution, linear link function and AR(1) correlation was used to compare the mean of value of titers, detected by ELISA, between treatment and control heifers at each collection period during the study while accounting for repeated measures from individual heifers. The margins and pair-wise comparison of the interaction term were performed as model post-estimations and were reported as descriptive statistics after exponentiation and comparisons between treatment groups by the time of titers measuring. The same model and the similar actions were performed to compare the mean of value of titers, detected by ELISA after exposure, between heifers aborted during the study and those that did not [37].

All models were assessed for outliers and goodness of fit. Differences were considered statistically significant when $P < 0.05$ [29].

3.4. RESULTS

3.4.1. Heat detection and bull exposure results

Observation of the Heatmount detectors suggested that all heifers expressed estrus during the first 12 days of bull exposure, 38 out of 50 heifers expressed estrus during the first 7 days and there was no difference between the treatment groups (Treatment (Tx)=19/25 (76%), Control

(C)=19/25 (76%); OR=1.00, 95% CI: 0.22–4.50; P=0.99). Observations of chin-ball markers suggested that all heifers were mounted by bulls at least once during the first 17 days of exposure, 46 out of 50 heifers were mounted in the first 12 days of exposure and there was no significant difference by treatment (Tx=22/25 (88%), C=24/25 (96%); OR=0.31, 95% CI: 0.01–4.22; P=0.61).

There was also no difference by treatment groups in the median number of days from the beginning of the study to when heifers expressed the first estrus (C=5, IQR: 2–7, Tx=4, IQR: 2–6; P=0.48). Similarly, the difference was not detected in median number of days from the beginning of the study to when heifers were mounted by bulls for the first time (Tx=6, IQR: 4–8, C=6, IQR: 2–8; P=0.35).

Three bulls, known to be infected with BGC, were used in this study: “19U”, “953” and “TD” (Table 3.4). Initially, “19U” and “953” were exposed to the heifers; however, bull “953” developed a toe abscess, was removed on day 13 and replaced by “TD”. During the study 44/50 (88%) of heifers were exposed to bull “19U”, 42/50 (84%) – to bull “953”, and 34/50 (68%) – to bull “TD”. Overall, 45/50 (90%) of heifers were mounted by more than one bull during the exposure and only 5 heifers were exposed to just one bull during the study (one to bull “19U”, four to “953”, and none to “TD”).

Based on observations of chin-ball markers in the first 12 days of exposure, bull “19U” demonstrated less breeding activity than “953” (“19U”=11%, “953”=23%; OR=0.38, 95% CI: 0.29–0.52; P<0.001), but there was no difference between bulls, detected during days 13–21, when “19U” was compared to “TD” (“19U”=19%, “TD”=21%; OR=0.85, 95% CI: 0.65–1.13; P=0.26). However, every bull in this study demonstrated similar exposure in the treatment and

control groups both on days 1–12 (“19U”: Tx=11%, C=10%, OR=1.11, 95% CI: 0.65–1.91, P=0.69; “953”: Tx=21%, C=24%, OR=0.86, 95% CI: 0.57–1.29, P=0.47) and on days 13–21 (“19U”: Tx=17%, C=20%, OR=0.85, 95% CI: 0.49–1.47; P=0.56; “TD”: Tx=23%, C=19%, OR=1.27, 95% CI: 0.75–2.16; P=0.37) of this study.

3.4.2. Results of preputial samples culture, confirmed by conventional PCR

Table 3.4 summarized the preputial culture results of the 3 bulls and the time period, each bull was exposed to the heifers. From 9 weeks before exposure to the heifers, all 3 bulls showed variable Cfv status, based on weekly culture and real-time PCR of preputial samples. Preputial samples of the bulls collected in 2, 4, 17 and 21 weeks after exposure confirmed that bull “19U” continued to be positive after the study.

Bull “19U” was treated for foot rot in week 1, but was sound within 2 days of application of a topical oxytetracycline foot bandage. This bull was confirmed as cultured positive on all 5 tests prior to heifer exposure and 3 after the start of heifer exposure. Bull “953” was replaced by “TD” in week 2. Despite remaining “physically sound” bull “TD” had culture and real-time PCR negative results for all but 1 test during the heifer exposure.

Overall, 21/39 (54%) of preputial samples collected from the bulls during the study were cultured negative. The bulls were tested once two weeks after breeding and smegma samples results were culture and real-time PCR negative. The bulls were subsequently retested on three occasions over the next 5 months with no further breeding exposure, and only one of them (“19U”) had culture and PCR positive results (Table 3.4). All three bulls were classified as satisfactory based on breeding soundness examinations before and after exposure to the heifers.

Bull ‘19U’ was retained at the research farm and demonstrated culture and real-time PCR positive results in the spring of 2014.

3.4.3. Results of vaginal mucus culture, confirmed by conventional PCR

The first culture-positive vaginal mucus samples were found two weeks after the start of bull exposure. The proportion of heifers in each treatment group with Cfv, isolated from their vaginal mucus, is summarized by week in Figure 1. There was no significant difference in the isolation of Cfv from vaginal mucous in the treatment and control groups in weeks 2 to 7, 9, 10, 12, 14, and 16 ($P>0.17$). The only significant difference between treatment groups was in week 8 (Tx=1/25 (4%); C=7/25 (28%); OR=0.11, 95% CI: 0.01–0.99; $P=0.04$) (Supl. Table 3.1).

During the study, 43 out of 50 heifers (86%) had at least one vaginal mucus sample, cultured positive for Cfv. There was no significant difference in risk of having at least 1 positive vaginal mucus culture between treatment groups (Tx=23/25 (92%); C=20/25 (80%); OR=2.87, 95% CI: 0.41–32.7; $P=0.42$) and between heifers, mounted by different bulls (“19U”=38/44 (86%); “953”=35/42 (83%); “TD”=31/34 (91%); $P=0.63$). Five of the 43 heifers (12%) cultured positive only once during the study period. There were five heifers that were mounted only by one bull during the study, based on observations of chin-ball markers and all of them were cultured positive for Cfv 2–7 times.

There was no difference in the median number times that heifers cultured positive for Cfv from vaginal mucus samples within each treatment group (C=3, IQR: 1–5; Tx=2, IQR: 2–3; $P=0.24$) and between heifers mounted by different bulls (“19U”=2, IQR: 1.5–4; 953=2, IQR: 1–4; “TD”=2, IQR: 1–4; $P=0.95$). There was also no significant difference in the median number days

from the start of exposure to when treatment and control heifers were first time cultured positive for Cfv from vaginal mucus (C=35, IQR: 21–42; Tx=28, IQR: 21–35; P=0.67).

3.4.4. Pregnancy diagnosis by serum PSPB and ultrasonography

The proportion of heifers, diagnosed pregnant by ultrasound and serum PSPB, by week and treatment group are summarized in Figures 3.2a and 3.2b. There was good agreement ($\kappa=0.840$, 95% CI: 0.839–0.841) between the two methods of detecting pregnancy. The first increase in serum level of PSPB above cut-off point (Table 3.3) was detected in week 4 of exposure and first pregnancy was detected by ultrasound in week 5.

The earliest pregnancy, diagnosed by ultrasound, was 25 days. We were able to estimate pregnancy term, detected by PSPB by comparing to ultrasound results of the same or consequent week in 14 out of 21 heifers, diagnosed pregnant during the study (1/14 was estimated 12 days; 5/14: 23–25 days; 8/14: 26–32 days). Therefore, the earliest pregnancy, detected by increased serum level of PSPB and estimated by comparing to subsequent results of ultrasound was 12 days.

The proportion of heifers, diagnosed pregnant by serum level of PSPB, did not differ between treatment and control groups for any week during the study ($P>0.31$) (Figure 2a, Supl.Table 3.2a). Similar findings were noted when the pregnancy was detected by serum ultrasound ($P>0.31$) (Figure 2b, Supl.Table 3.2b).

During the study 21/50 (42%) of heifers were detected pregnant at least once and there was no significant difference between treatment groups (Tx=12/25 (48%); C=9/25 (36%); OR=1.64, 95% CI: 0.46–5.94; P=0.57). There was no difference between the treatment and control groups in the median number days to first pregnancy either in all heifers, detected pregnant during the study

(C=47, IQR: 17–52; Tx=19, IQR: 10–38; P=0.30) or in those that remained pregnant at the end of the study (C=17, IQR: 9–35; Tx=16, IQR: 7–40; P=0.70) and animals that never aborted, maintaining active pregnancy (C=17, IQR: 2–54; Tx=40, IQR: 7–52; P=0.83), based on ultrasonography.

Fifteen of 21 pregnant heifers lost their pregnancy (71%), but 6 of the 15 heifers, who lost their pregnancy, became pregnant again by the end of the study. Therefore, 6 out of 12 pregnant at the end heifers (50%) aborted earlier and other six maintained active pregnancy (three in each treatment group). We estimated that 42% of abortions happened before day 25 of gestation, 47% between days 25–35, and 11% in days 45–55 of pregnancy. Every heifer, aborted during the study, aborted only once accordingly to the data from our weekly pregnancy checking. There was no significant difference in pregnancy loss rates by treatment group (Tx=75%; C=67%; OR=1.48, 95% CI: 0.76–2.87; P=0.28). However, aborted animals had more frequent positive vaginal mucous culture results (median=2, IQR: 2–4) than heifers that did not abort (median=0.5, IQR: 0–1) (P=0.01).

Overall, 12 heifers were pregnant at the end of the study: 5 were from the control group and 7 from the treatment group. The 12 pregnant heifers at the end had less frequent cultured positive results for Cfv in vaginal mucus (median 1.5, IQR: 0.5–2) as compared to the 38 non-pregnant heifers (median 3, IQR: 2–4) (P=0.002). The 5 heifers, pregnant at the end of the study, in the control group cultured positive a median of 1 time (IQR: 0–2), compare to 4 times for 20 non-pregnant heifers in this group (IQR: 2–6) (P=0.04). The 7 heifers from treatment group, who were pregnant at the end, were cultured positive a median of 2 times (IQR: 1–2), compare to 3 times for 18 non-pregnant heifers from the same group (IQR: 2–3) (P=0.01). However, there was

no difference between treatment groups in number of culture positive samples in non-pregnant heifers at the end of the study (C: median=4, IQR: 2–6; Tx: median=3, IQR: 2–3; P=0.14).

3.4.5. *C. fetus* antibody titer serology

All blood samples, collected from the heifers 7 weeks before and in week 0 of exposure (3 weeks before and 4 weeks after vaccination), MAT titers values for *C. fetus* were less than 2, which are considered negative. In week 2 of exposure (week 6 after vaccination), 5 samples were positive (titer>4), and in week 9 only 2 heifers were positive. One of 7 positives showed MAT titres of 32, two were 64, three were 128, and one was 256. Samples, collected in week 16, were all negative. Four positive samples were from the treatment group and three were from the control group. No significant difference was found by treatment in either the maximum detected titers (Tx: median=2, min=2, max=128; C: median=2; min=2, max=256; P=0.57) or the proportion of positive titers during the study (Tx=16%; C=12%; OR=1.40, 95% CI: 0.58–3.44; P=0.99).

ELISA titers for *C. fetus* results are summarized in the Table 3.5. In a model, including treatment, time, and a treatment/time interaction, there was no difference between treatment groups in ELISA titres mean before vaccination and exposure (Tx=74, 95% CI: 54–102; C=87, 95% CI: 61–125; P=0.52). However, there was significant difference between treatment groups after vaccination (P<0.02). By week 4 after the second vaccination and immediately before exposure, the mean in the treatment group was 3 times higher than of control group (Tx=486, 95% CI: 412–572; C=169, 95% CI: 119–241; P<0.001). A similar difference was observed in week 13 post-vaccination (week 9 of exposure) when titer mean value was the highest during the study (Tx=659, 95% CI: 478–909; C=236, 95% CI: 139–401; P<0.001). Titer value mean in

treatment group was 2 times higher than in control group in weeks 6 ($P=0.02$) and week 20 ($P=0.003$) post-vaccination (weeks 2 of and 7 after exposure) (Table 3.5).

Comparing ELISA titers means between the heifers that aborted during the study with those that did not, there was no significant difference either in week 2 (aborted=122, 95% CI: 74–200; non-aborted=283, 95% CI: 129–528; $P=0.07$), week 9 (aborted =382, 95% CI: 232–628; non-aborted =317, 95% CI: 145–696; $P=0.70$), or week 16 (aborted =153, 95% CI: 93–252; non-aborted =71, 95% CI: 32–155; $P=0.10$) after exposure.

3.5. DISCUSSION

Herein we compared the pregnancy and infection rates between heifers, vaccinated with a polyvalent vaccine, containing *C. fetus* antigen, to those, vaccinated with a comparable polyvalent product that did not contain *C. fetus* antigen. Heifers were paired by body weight and then randomized within each pair into treatment or control group to balance the starting weight of the animals and minimize the influence of differences in maturity on cyclicity and immunity. Animals in treatment and control groups expressed estrus and were adequately naturally challenged. There was no significant difference detected between treatment groups in percentage of heifers that became infected with CfV, proportion of heifers that became pregnant, or difference in days to pregnancy. However, serum concentration of *C.fetus* antibodies, detected by ELISA, was significantly higher in treatment group compared to control animals.

Our visual heat detection and chin-ball markers results suggested that all heifers expressed estrus and were mounted by bulls at least once by the day 17 of exposure. This was equally distributed between two treatment groups. There was also no difference between treatment groups in the time of expressing the first estrus and first bull mounting.

We had two naturally infected bulls with the heifers through all 63 days of our study. One of the bulls demonstrated significantly higher mounting activity, compare to the other, in the first 12 days according to the chin-ball marker results. The second bull had been treated for footrot in the first week but appeared to respond well to topical therapy. When the more dominant bull was replaced due to a toe abscess, there was no difference in breeding activity between two remaining bulls from days 13 to 21. However, every bull had similar breeding activity in both heifer treatment groups of heifers during the study.

After exposure to the heifers, the bulls became less likely to culture positive for Cfv, and only the one bull, that was with the heifers for the full study, cultured positive consistently during and after the study. There was an older study that documented reduced shedding of *T. foetus* in bulls after active breeding and hypothesized that this could happen due to mechanical removal microorganisms from penile mucosal membranes during coitus [38]. For the same reason a bull cannot be judged free from BGC infection if he fails to infect a single virgin heifer during natural service [39]. In case of *T. foetus* it is not usually found in preputial samples for several days subsequent to coitus [38]. Another suggested reason could be contamination with other organisms that can overgrow the pathogen of interest during culturing or other DNA can be inhibitors for specific primers during PCR. The possible causes of contamination of preputial cavity could be masturbation or intermittent prolapsed of mucosal membranes [38]. However, these explanations are not conclusive since no publications were found testing such hypothesis.

Lameness that occurred in two of the bulls remained a concern in terms of overall pregnancy outcomes. However, all bulls had satisfactory breeding soundness exams, such as physical soundness, scrotal circumference and semen examination [31], and demonstrated good breeding activity at the beginning and the end of the trial. The chin-ball marker results and culture-positive

rate for Cfv from vaginal mucus in both treatment groups suggested that lameness did not impede breeding and adequate challenge.

One of bulls was cultured positive for Cfv only once during the study which might present a concern about adequate challenge of heifers. However, our chin-ball markers results demonstrated that all heifers, mounted by that bull, were also mounted by other bulls.

Overall, 90% of heifers were mounted by more than one bull during the exposure. When heifers, mounted by different bulls, were compared in the rate of cultured positive for Cfv at least once during the study, it was similarly high (83–91%). Similarly, heifers were cultured a median of 2 times positive for Cfv during the trial, no matter by which bull they were mounted. Five heifers that were mounted only by one bull during the exposure period were cultured positive several times during the study, suggesting a satisfactory and uniform challenge.

In this study, the Cfv infection challenge was achieved as 86% of heifers cultured positive for Cfv at least once during the study and there was no significant difference between treatment groups but 95% CI of the Odd Ratio was of huge range (0.41–32.7). However, all other 95% CI of ORs, combined during this study were within acceptable limits (0.01–5.94), suggesting that an issue with “study power” was not a concern; however, it would be prudent to increase the sample size in future related studies.

The difference in “pathogen load” between groups was not assessed since colonies count was not performed. This could be considered as one of weaknesses of this study; however, accordingly to subsequent conventional PCR results not all submitted colonies were of Cfv. Therefore, quantitative analysis of plates was not probably appropriate in this study because some colonies might be of Cff and since some plates contained quite big number of colonies it would be

difficult to perform Gram-stain and PCR from all of them. The other issue with quantitative analysis by colony counts or real-time PCR is that we do not have a consistent sample by weight or volume.

Given that most heifers were cultured positive at least once, and that only 12% were positive only once during the study period, it would either appear that transient infections with Cfv were uncommon or suggestive of poor culture sensitivity in our study. There were no reports found about possibility of BGC transient infection in female cattle. Moreover, these outcomes indicate adequate challenge and exposure of most heifers to more than one infected bull in our study.

The highest proportion of vaginal mucus samples with Cfv isolated in our study occurred from weeks 4 to 6. The results of another similarly organized trial, using commercial and experimental polyvalent vaccines, demonstrated the highest proportion of positives in weeks 7–15 [24]. Such discrepancy was most likely due to the fact that half of the animals of the earlier trial were also challenged at day 39 of breeding season by an intravaginal instillation of Cfv. However, when control groups of two studies are compared, the proportion that cultured positive in weeks 4–6 was similar in both studies (around 25–40%).

Uterine secretions in cattle are characterized by greater concentrations of IgG than IgA [reviewed in 35]. In some animal models, vaginal or nasal application of antigens resulted in optimal uterine responses, but this has not been established for cattle [reviewed by 40]. If the latter were demonstrated in cattle, the goal of Cfv vaccination would be stimulation of systemic IgG production for subsequent secretion within the uterine mucus.

The efficacy of commercially available polyvalent vaccines against BGC could also depend on the adjuvants used. The majority of vaccines against *C. fetus* are prepared with oil or aluminum

hydroxide adjuvants [11, 13, 14, 21, 41]. Aluminum hydroxide is able to induce a good antibody (Th2) response; however, it has little capacity to stimulate cellular (Th1) immune responses [42] due to its poor adjuvant effect on polysaccharide antigens, and can increase IgE production [43]. The most effective vaccines are prepared with oil adjuvants [reviewed in 13]. They stimulate strong antibody and cell-mediated responses but may cause a granuloma at the injection site [13, 24, 42]; however, newer oil containing adjuvants are much improved [13, 42].

In our study, pregnancy rates were low for both treatment groups (Tx=48% and C=36%). Beef heifers commonly have pregnancy rates >95% after 63 days of bull exposure [44]. However, the percentage of non-pregnant heifers was consistent with a reported natural outbreak of BGC in western Canada [45].

The overall proportion of pregnant heifers was not significantly different between the two vaccination groups and was in agreement with another vaccine trial with two commercial vaccines [22]. In that study 16 heifers were vaccinated parentally twice, 6 and 3 weeks before breeding, with a polyvalent vaccine containing Cff and Cfv and subsequently served by an infected bull for 60 days. The result was limited because of the low number of animals, but the two commercial vaccines were not efficient in improving pregnancy rate. Another reason of poor results from that study was apparently poor immune response to vaccination. The increase of antibodies to *C. fetus* was not detected post vaccination either in vaginal mucus samples by IFAT or in serum by ELISA [22]. Therefore, it is difficult to say whether small sample size was the main reason for the negative results of vaccination in that trial.

Another study compared polyvalent vaccines that contained *Trichostrongylus axei*, Cfv, as well as other common pathogens, given subcutaneously, and an experimental vaccine, applied

subcutaneously and intravaginally, that contained only *T. foetus* and *C. fetus* subspecies, originally isolated from a cow with pyometra, together with an oleo adjuvant. All heifers were exposed to naturally infected bulls over a 90-day period. The results indicated that the experimental vaccine yielded superior protection (significantly higher pregnancy rates) compared to the commercial vaccine [24].

When considering the pregnancy rates in our study, it is important to recognize that the heifers remained exposed to the bulls long enough to get pregnant, abort and become pregnant again. A total of 50% of the heifers that were pregnant at the end of the study, had aborted earlier. Only 6 heifers never aborted during the study and maintained pregnancy up to the end.

Pregnancy rates in both treatment groups were very poor by industry standards. All bulls used in our study had undergone a breeding soundness examination before the trial began and demonstrated satisfactory results. There is no published evidence to suggest that BCG negatively effects spermatozoa. Actually, there is a study demonstrated that *T. foetus* is able to adhere to sperm cells resulting in agglutination, immune reaction and reducing motility of sperms [46, 47]. In addition, *T. foetus* expresses a cystein protease that responsible for cytotoxic effect against sperms [47]. However, there is no data found determined the effect of Cf_v. The Cf_v has not been isolated from accessory glands or epididimis and its presence in bull ejaculate is believed to be due to contamination of preputial cavity [48, 49]. Therefore, Cf_v would rather not influence sement quality.

In this study, the time to first detected pregnancy in all heifers, detected pregnant during the study, those that remained pregnant at the end of the study, and pregnant heifers that never

aborted during the study, did not differ by treatment. Therefore, vaccination against BGC with polyvalent product did not significantly shorten time to conception in our study.

Ultrasound and serum PSPB gave similar pregnancy results and similar kappa values in our study, compare to published data [50]. Most of the disagreements were in the first weeks of the experiment. Early pregnancies could cause an increased concentration of PSPB, but could be missed by ultrasound. PSPB has been reported to detect as early as 16 days of pregnancy [51], which is earlier than the 26–28 days of detection for ultrasound [52]. In this study we utilized the kit that should detect pregnancy from 28 days after breeding with 99% of accuracy [34]. However, we estimated the detected pregnancies in our study by PSPB from 12 days. PSPB level also remained elevated for a period after pregnancy loss, which could potentially result in misclassification, but we also used ultrasound and compared the results. In this study, PSPB blood level was only elevated once in two animals at the beginning of the trial, that never consequently confirmed by ultrasound. Therefore, in most cases we were able to estimate pregnancy term when detected by the serum level of PSPB.

More than half of the heifers in this study never detected pregnant by ultrasound or serum PSPB. The question remains, were they never pregnant or did they have early embryonic loss? It is often cited that Cfv results in pregnancy loss at 60–75 days post-breeding [1, 10], but based on earlier methods of detection (ultrasound and PSPB) losses could occur earlier. In our study the earliest pregnancies, detected by ultrasound, were about 25 days that were confirmed by serum level of PSPB. Sometimes pregnancies were detected by PSPB and were not confirmed by ultrasound and if not detected at the next week we estimated that abortion happened before 25 days of pregnancy. We estimated that most abortions happened before day 35 of gestation. Abortion rates did not differ by vaccination group.

We could not determine whether immunity improved after abortion (reconvalescent immunity), based on MAT, since a low number of titer-positives was detected throughout the study. However, the ELISA results allow the conclusion that there was no significant difference in serum concentrations of IgG to *C. fetus* between heifers that aborted during the study and those that did not.

Very few MAT-positive titers were detected during our study, resulting in no significant differences by week or treatment. After systemic immunization, antibodies of IgG and IgM classes were commonly detectable in serum, while IgA and IgG1 were transported across the epithelium into genital secretions [53]. ELISA is suggested as the best monitoring test for systemic antibodies against *Campylobacter* spp [54]. In our study, significant rise of systemic IgG antibodies to *C. fetus* titers in treatment group was detected by ELISA that was significantly higher than in control group. In contrast, ELISA did not detect any significant rise in systemic IgG antibody titers after systemic immunization in another vaccine trial where aluminum hydroxide adjuvant was used; there was only slight increase in antibody concentrations immediately after the breeding period. This may have been due to natural exposure to Cfv antigens in the semen of the infected bull [22].

In our study significant rises in antibody titers were detected by ELISA in treatment group by week 4 after vaccination, right before heifers were exposed to bulls. During the bull exposure period antibody titers in the treatment group were significantly higher than that of the control group. This suggests that ELISA could be a more sensitive method to monitor the systemic antibody response to Cfv after vaccination than the MAT technique since MAT measures only surface antigens but ELISA is able to detect internal and external antigens of bacterial cells [54]. It has been suggested that antigenic differences between strains may cause the lack of

effectiveness of the vaccines [24]. However, the commercial vaccine was able to induce antibody responses, detected by ELISA, that were significantly higher after vaccination in the treatment group of heifers, compared to the control group. Alternatively, even though the antibody responses in treatment groups were statistically higher, they may have been higher if the animals had been immunized with antigens more similar to the infecting strain.

It was suggested that aluminum hydroxide generally induces an immunity of shorter duration than an oil adjuvant vaccine [55]. Using oleo adjuvant induced significant antibody response that was detected by *C. fetus*-IgG ELISA test [24]. In our study, the serum level of antibodies to *C. fetus* was continuously more than 2 times higher in treatment group of heifers, compared to control animals, for about 5 months post vaccination (time of the last sampling). Unfortunately, we could not determine whether the antibody level was increased in the subsequent months. Sampling was stopped at week 12 of bull exposure as interpreting the results became more difficult due to the confounding of natural infection as heifers aborted and became pregnant again.

3.6. CONCLUSIONS

Vaccination of heifers with a polyvalent commercial vaccine, containing *C. fetus* antigen, according to the label did not significantly reduce infection rates or improve pregnancy rate when compared to heifers vaccinated with a similar polyvalent commercial vaccine that did not contain *C. fetus* antigen, when exposed to Cf_v-infected bulls.

^aGoodale Research Farm, University of Saskatchewan

^bEasi-Scan II (BCF Technology, Minneapolis, MN)

^cVista 5 L5 (Merck Animal Health, Kirkland, PQ)

^dVista 5 VL5 (Merck Animal Health, Kirkland, PQ)

^eFusogard

^fKow-Ball Marker, Western Sales, Brush, CO, Item # 50240

^gKamar Products Inc., Zionsville, IN

^hContinental Plastics, Vetsource Canada, Cambridge, ON; Item #E6-5415

ⁱContinental Plastics, Vetsource Canada, Cambridge, ON; Item #B6-4775

^jBecton Dickinson

^kMillipore, Billerica, MA, USA

^lGasPak™ EZ Campy Pouch™ System (BD diagnostics, Mississauga, ON, Canada)

^mBioPRYN, BioTracking, Moscow, ID

ⁿPrairie Diagnostic Services (Saskatoon, SK)

^oZoetis Animal Health (Kalamazoo, MI)

^pStata 13.1, Special Edition, Stata Corp., College Station, TX

Table 3.1. Peer-reviewed studies regarding vaccination for Bovine Genital Campylobacteriosis

Study	Vaccine type	Animals, sex and number	Vaccine route	Challenge	Authors' conclusion
Clark et al, 1968 [15]	Monovalent, experimental	Bulls, n=4	Systemic	Culture infusion	Effective
Allan and Mutch, 1971 [26]	Monovalent, commercial	Heifers, n=334	Systemic	Natural exposure	Effective
Allan, 1972 [18]	Monovalent, commercial	Heifers, n=261 Bulls, n=70	Systemic	Natural exposure	Effective Questionable
Clark et al, 1972 [27]	Monovalent, experimental	Heifers, n=125	Systemic	Natural exposure Culture infusion	Effective
Clark et al, 1972 [28]	Monovalent, experimental	Heifers, n=80	Systemic	Natural exposure Culture infusion	Effective
Clark et al, 1972 [20]	Monovalent, experimental	Cows, n=102	Systemic	Natural exposure	Effective
Bouters et al, 1973 [14]	Monovalent, experimental	Bulls, n=321	Systemic	Natural exposure	Effective
Shurig et al, 1975 [25]	Monovalent, experimental	Heifers, n=16	Systemic	Culture infusion	Effective
Clark et al, 1976 [19]	Monovalent, experimental	Heifers, n=80 Bulls=13	Systemic	Culture infusion	Effective
Johns et al, 1977 [21]	Monovalent, commercial	Heifers, n=33	Systemic	Culture infusion	Effective
Shurig et al, 1978 [23]	Monovalent, experimental	Heifers, n=32	Systemic	Culture infusion	Effective
Clark et al, 1982 [11]	Monovalent, commercial	Bulls, n=24	Systemic	Culture infusion	Effective
Vasquez et al, 1983 [17]	Monovalent, commercial	Bulls, n=12	Systemic	Culture infusion Natural exposure	Effective
Eaglesome et al, 1986 [16]	Monovalent, experimental	Heifers, n=46 Bulls, n=4	Systemic	Natural exposure	Effective
Cobo et al, 2003 [22]	Polyvalent, commercial	Heifers, n=24	Systemic	Natural exposure	Questionable
Cobo et al, 2004 [24]	Polyvalent, commercial	Heifers, n=31	Systemic	Natural exposure Culture infusion	Both effective, Experimental–superior effect
	Monovalent, experimental	Heifers, n=31	Systemic Local	Natural exposure Culture infusion	
Foscolo et al, 2005 [15]	Monovalent, experimental	Bulls, n=27	Systemic	Naturally infected before trial	Questionable

Table 3.2. Timeline for sample and data collection. Including dates of vaccination, bull exposure, preputial sampling, vaginal mucus collection, blood collection, transrectal ultrasonography and visual heat detection

Date	Day	Wk	Vaccination		Bull Exposure	Sample and Data Collection					
			Vista 5 VL5	Vista 5 L5		Preputial Sample	Vaginal Mucus	Blood		Trans -Rectal Ultrasound	Visual Heat Detection
20-May-13	-49	-7	Yes	Yes		Yes		Yes			
10-Jun-13	-28	-4	Yes	Yes		Yes	Yes			Yes	
08-Jul-13	0	0			Yes	Yes		Yes		Yes	Yes
15-Jul-13	7	1			Yes	Yes	Yes				Yes
22-Jul-13	14	2			Yes	Yes	Yes	Yes	Yes		Yes
29-Jul-13	21	3			Yes	Yes	Yes		Yes		Yes
05-Aug-13	28	4			Yes	Yes	Yes		Yes	Yes	
12-Aug-13	35	5			Yes	Yes	Yes		Yes	Yes	
19-Aug-13	42	6			Yes	Yes	Yes		Yes	Yes	
26-Aug-13	49	7			Yes	Yes	Yes		Yes	Yes	
02-Sep-13	56	8			Yes	Yes	Yes	Yes	Yes	Yes	
09-Sep-13	63	9			Yes	Yes	Yes		Yes	Yes	
16-Sep-13	70	10					Yes		Yes	Yes	
23-Sep-13	77	11								Yes	
30-Sep-13	84	12					Yes			Yes	
07-Oct-13	91	13								Yes	
14-Oct-13	98	14					Yes			Yes	
21-Oct-13	105	15								Yes	
28-Oct-13	112	16					Yes	Yes		Yes	

Table 3.3. The optical density (OD) values of PSPB concentration for determining Pregnant/Non-Pregnant status in BioPRYN assay

Not Pregnant	Low Recheck	Cutoff	High Recheck	Pregnant
OD < 0.135	OD = 0.135 to 0.15	0.15	OD = 0.15 to 0.21	OD > 0.21

Table 3.4. Summary of the bull preputial culture, confirmed by multiplex conventional PCR, and real-time PCR results for *Campylobacter fetus* subsp. *venerealis* before, during and after the periods of exposure to heifers

Week		Bulls								
		953			19U			TD		
		Results		Exposure	Results		Exposure	Results		Exposure
		Culture	Real-time PCR		Culture	Real-time PCR		Culture	Real-time PCR	
-9	Before breeding	+	+	n/a*	+	+	n/a	+	+	n/a
-8		+	+	n/a	+	+	n/a	-	+	n/a
-7		+	+	n/a	+	+	n/a	+	+	n/a
-6		+	+	n/a	+	+	n/a	+	+	n/a
0	During breeding period	+	n/a	+	+	n/a	+	+	n/a	-
1		-	n/a	+	-	n/a	+	-	n/a	-
2		-	-	-	-	+	+	-	+	+
3		n/a	n/a	-	-	-	+	-	-	+
4		n/a	n/a	-	-	+	+	+	+	+
5		n/a	n/a	-	+	+	+	-	-	+
6		n/a	n/a	-	+	+	+	-	-	+
7		n/a	n/a	-	+	+	+	-	-	+
8		n/a	n/a	-	-	-	+	-	-	+
9		n/a	n/a	-	-	-	+	-	-	+
+2	After breeding	+	+	n/a	+	-	n/a	-	-	n/a
+4		-	-	n/a	-	-	n/a	+	-	n/a
+17		-	-	n/a	+	+	n/a	-	-	n/a
+21		-	-	n/a	+	+	n/a	-	-	n/a

*n/a- not applicable

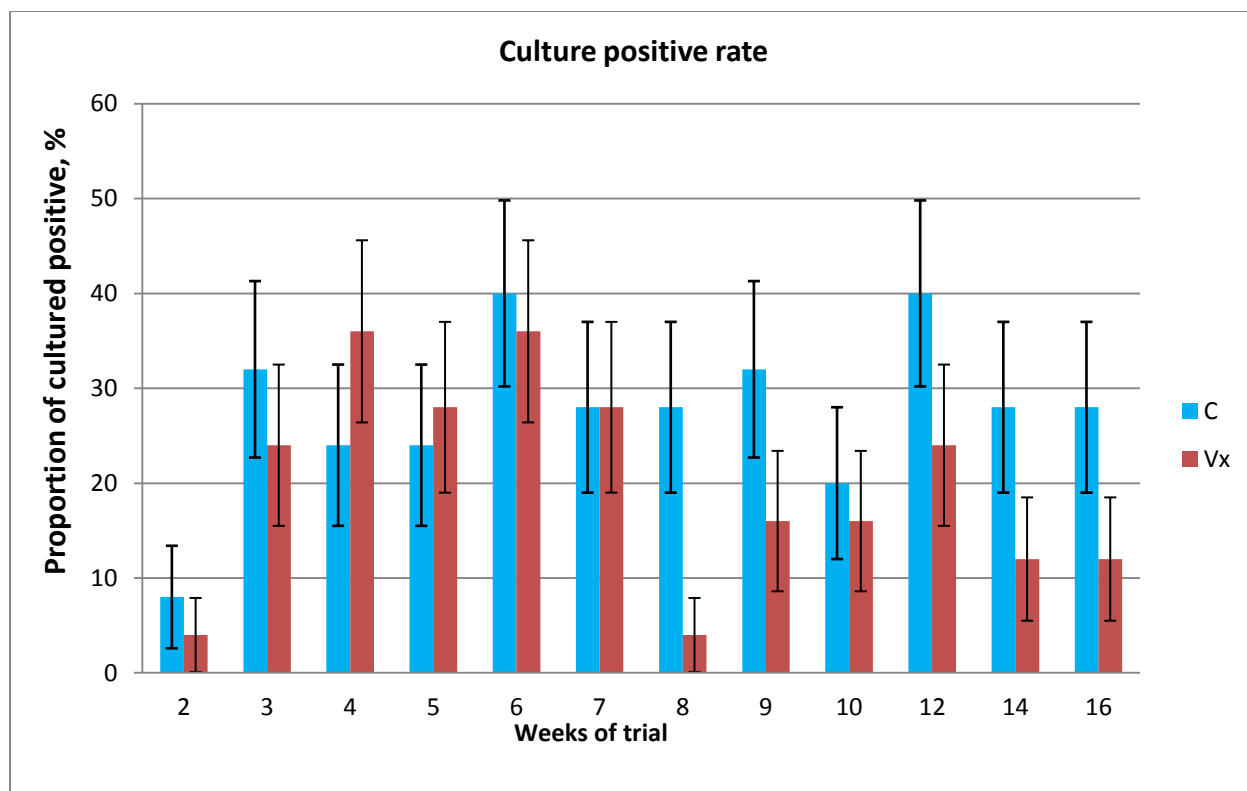


Figure 3.1. Percentage of vaginal mucus samples, cultured positive for Cfv and confirmed by multiplex conventional PCR, by week and treatment (errors bars represents standard errors (SE))

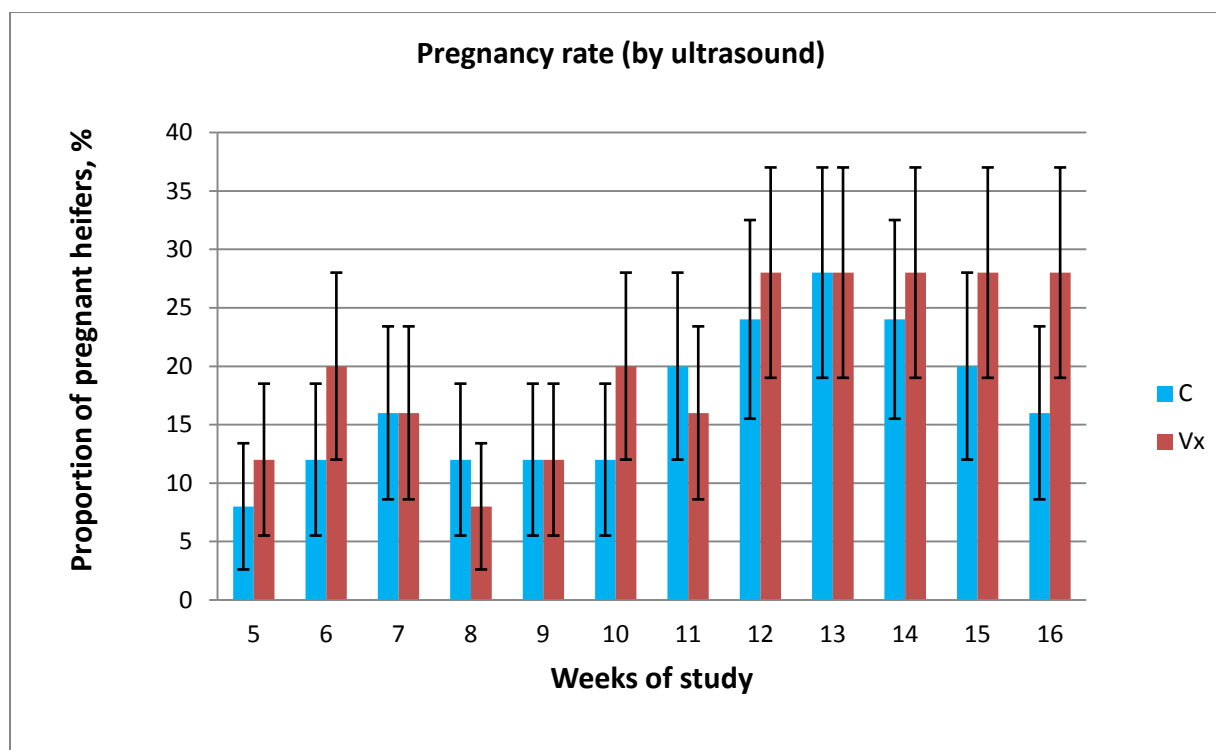
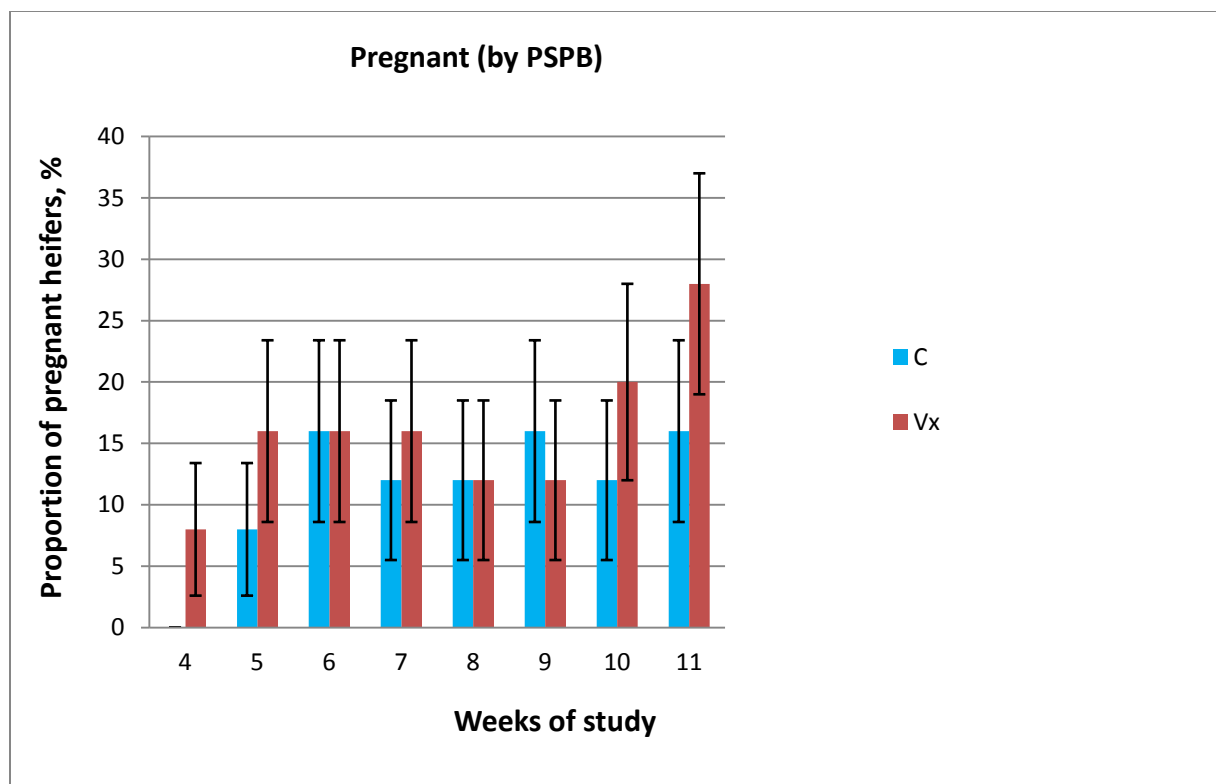


Figure 3.2a and 3.2b. Proportion of detected pregnant in both groups during the trial by week and treatment (errors bars represents SE)

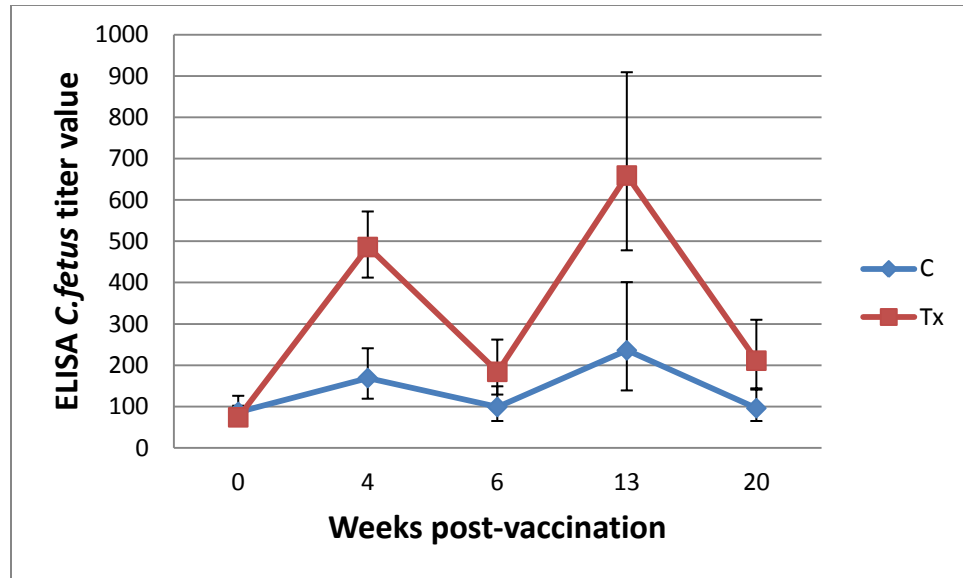


Figure 3.3. ELISA antibody to *C. fetus* titers results by week of study and treatment groups
(errors bars represents 95% CI)

Supplemental Table 3.1. Comparison of samples, cultured positive for Cfv and confirmed by multiplex conventional PCR, by treatment groups

Weeks of study	Cultured positive, %				OR	95% CI	P-value*
	Tx (n=25)		C (n=25)				
	Proportion	95% CI	Proportion	95% CI			
2	4	0–12	8	0–19	0.48	0.04–5.65	0.56
3	24	7–41	32	14–50	0.67	0.19–2.33	0.53
4	36	17–55	24	7–41	1.78	0.52–6.09	0.36
5	28	10–46	24	7–41	1.23	0.35–4.37	0.75
6	36	17–55	40	21–59	0.84	0.27–2.65	0.77
7	28	10–46	28	10–46	1.00	0.29–3.44	0.99
8	4	0–12	28	10–46	0.11	0.01–0.95	0.04
9	16	2–30	32	14–50	0.40	0.10–1.57	0.19
10	16	2–30	20	4–36	0.76	0.18–3.25	0.71
12	24	7–41	40	21–59	0.47	0.14–1.60	0.23
14	12	1–25	28	10–46	0.35	0.08–1.55	0.17
16	12	1–25	28	10–46	0.35	0.08–1.55	0.17

*Represents difference between treatment and control groups by week in a model accounting for repeated measures and including treatment, time, and treatment*time interaction.

Supplemental Table 3.2a. Comparison of detected pregnant (by PSPB) by treatment groups

Weeks of study	Detected pregnant by PSBP, %				OR	95% CI	P-value*
	Tx (n=25)		C (n=25)				
	Proportion	95% CI	Proportion	95% CI			
4	8	0–19	0	0	n/a	n/a	n/a
5	16	2–30	8	0–19	2.19	0.36–13.21	0.39
6	16	2–30	16	2–30	1.00	0.22–4.53	0.99
7	16	2–30	12	1–25	1.40	0.27–7.00	0.68
8	12	1–25	12	1–25	1.00	0.18–5.51	0.99
9	12	1–25	16	2–30	0.72	0.14–3.59	0.68
10	20	4–36	12	1–25	1.83	0.39–8.67	0.44
11	28	10–46	16	2–30	2.04	0.51–8.12	0.31

*Represents difference between treatment and control groups by week in a model accounting for repeated measures and including treatment, time, and treatment/time interaction.

Supplemental Table 3.2b. Comparison of detected pregnant (by ultrasound) by treatment groups

Weeks of study	Detected pregnant by ultrasound, %				OR	95% CI	P-value*
	Tx (n=25)		C (n=25)				
	Proportion	95% CI	Proportion	95% CI			
5	12	1–25	8	0–19	1.57	0.24–10.30	0.64
6	20	4–36	12	1–25	1.83	0.39–8.67	0.44
7	16	2–30	16	2–30	1.00	0.22–4.54	0.99
8	8	0–19	12	1–25	0.64	0.10–4.19	0.64
9	12	1–25	12	1–25	1.00	0.18–5.51	0.99
10	20	4–36	12	1–25	1.83	0.39–8.67	0.44
11	16	2–30	20	4–36	0.76	0.18–3.25	0.71
12	28	10–46	24	7–41	1.23	0.35–4.37	0.75
13	28	10–46	28	10–46	1.00	0.29–3.44	0.99
14	28	10–46	24	7–41	1.23	0.35–4.37	0.75
15	28	10–46	20	4–36	1.55	0.42–5.78	0.51
16	28	10–46	16	2–30	2.04	0.51–8.12	0.31

*Represents difference between treatment and control groups by week in a model accounting for repeated measures and including treatment, time, and treatment/time interaction.

Supplemental Table 3.3. Comparison of ELISA antibody to *C. fetus* titers results by treatment groups

Week after vaccination	Week of exposure	Treatment groups, n=25 per group						P-value*
		Tx (Vaccine with <i>C. fetus</i> antigen)			C (Vaccine without <i>C. fetus</i> antigen)			
		Mean	95% CI		Mean	95% CI		
0	-7	74	54	102	87	61	125	P=0.55
4	0	486	412	572	169	119	241	P<0.001
6	2	184	129	262	99	65	149	P=0.02
13	9	659	478	909	236	139	401	P<0.001
20	+7	211	144	310	96	65	141	P=0.003

*Represents difference between treatment and control groups by week in a model accounting for repeated measures and including treatment, time, and treatment/time interaction.

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4. GENERAL DISCUSSION AND FUTURE RESEARCH

4.1. GENERAL DISCUSSION

This thesis describes the comparison of diagnostic approaches to detect *Campylobacter fetus* subsp. *venerealis* (Cfv) and evaluates the efficacy of vaccination for Bovine Genital Campylobacteriosis (BGC). Different bovine vaginal mucus sample preparation methods for real-time PCR were compared in order to detect Cfv and the efficacy of a polyvalent commercial vaccine in preventing infection and reproductive loss in cattle was evaluated.

C. fetus is divided into two subspecies, Cff and Cfv, which are different in their epidemiologic and clinical importance; therefore, differentiation on the subspecies level is important. Conventional PCR, based on two primer pairs (one targets the carbon starvation gene in both subspecies, while another targets the *parA* gene in Cfv), had high agreement with other typing methods when done directly from colonies [1, 2]. A real-time PCR, targeting the *parA* gene in Cfv, was successfully utilized for bull preputial samples [3] but required adaptation of the sample preparation method on bovine vaginal mucus.

Innate immunity is a vital protection mechanism for exposed female cattle [4]. Acquired immune response is also important. Mucus membranes of the uterus are characterized by a greater IgG than IgA response [5]. Vaccine-induced immunity is usually effective but short-lived and annual booster is required [6]. The efficacy of vaccines depends on the antigens and adjuvants they contain [7]. There are relatively limited number of peer-reviewed studies about vaccination for BGC, most of which more than 30 years old and focused on monovalent experimental vaccination. Only couple more recent studies were found describing the effectiveness of commercial polyvalent vaccines. Since polyvalent commercial vaccines are more convenient to

use for beef producers than monovalent experimental vaccines, there is a need to better understand the efficacy of vaccination with a commercially available polyvalent vaccine.

The purpose of this chapter is to summarize the main findings obtained from the two studies in this thesis. The strengths and limitations, experienced during these studies will be discussed and potential improvement for future studies will be explored. Finally, necessary conclusions will be made.

In the first study's (Chapter 2) the results of Cfv real-time PCR were compared for bovine vaginal mucus samples, prepared using heat lysis and magnetic bead based sample preparation techniques, to results of direct, selective culture of the CVM. The sample processing method, designed and successfully applied for bull preputial samples [3], did not demonstrate adequate sensitivity when applied on vaginal mucus. All modifications of that heat lysis protocol demonstrated low sensitivity when were compared to culture confirmed by conventional PCR. The magnetic bead-based extraction method tended to be the comparatively more sensitive than heat lysis methods but required much of time and resources to perform.

One possible explanation for low sensitivity of the sample processing method, designed and successfully applied for bull preputial samples [3], could be different biophysical properties of vaginal mucus, compare to preputial scrape material. Nucleic acid isolation protocol, designed for preputial samples, contained several steps to eliminate possible inhibitors [3]. Even though, quantification analysis was not done, the modifications of heat lysis sample processing protocol, initially designed for prepuce samples, employing fewer dilution steps had greater sensitivity in this study.

The aqueous phase of bovine CVM contains low-molecular-mass compounds [8] and the gel phase is formed mainly by insoluble high-molecular-mass compounds [9]. The gel phase is probably responsible for high viscosity of CVM in certain stages of estrus cycle [10]. Properties of CVM depend on the stage of estrus cycle [9]: CVM becomes less viscous and more hydrated during the pre-ovulatory period [10] and vice versa in the luteal phase [10–12]. An inverse relationship between the dry matter content in CVM and the degree of crystallization was noted [11]. Reduction in the proportion of salts in aqueous phase leads to reduction of water content, consequent reduction in crystallization and relative increase of other salt components in the dry residue. At the estrus dry matter concentration is minimum and crystallization is maximum [12, 13]. Therefore, CVM often has gelly appearance due to either high viscosity or crystallization, and this does not depend on the stage of estrus cycle, during which sampling was performed.

Different DNA isolation protocols can be utilized on clinical samples. In one study, the VenSF/VenSR-based PCR on the samples, collected mainly during the estrous cycle, incubated in TEM at 37°C for 5 days and processed with CTAB, provided Cfv detection in 24% cases, compared to 2.8% by bacterial isolation in culture [13]. This large number of detected positives was most likely due to maintaining viability of the pathogen in that study. In our study, however, some modifications were made to the existing heat lysis isolation protocol, skipping some steps, in order to test a rapid sample preparation method that could be used on field samples. When modified protocols were compared to culture, the most adequate results were obtained by performing protocols with fewer dilution steps. The two most sensitive heat lysis isolation methods were consequently compared to a magnetic bead-based extraction method that described as demonstrated satisfactory results in different samples for a variety of pathogens [14–16].

All compared isolation methods had low sensitivity in relation to culture, confirmed by multiplex conventional PCR. That could be explained by several reasons. The first explanation is the possibility that culture, confirmed by conventional PCR actually was not the gold standard. However, we detected more positives by culture, confirming by conventional PCR, than by real-time PCR. On other hand, samples were kept under ideal conditions prior to culturing, which is not always practical. Another explanation for the low sensitivity of the tested methods is that the freeze-thaw process, which can disrupt cellular compartments [17], was repeated several times in our study. Therefore, further testing of the DNA isolation methods should be performed utilizing cryoprotectants or on fresh samples.

Theoretically, there might be a chance that results of our study were confounded by the fact that the primers for the PCR assay were not specific enough for Cfv. However, it is difficult to make any conclusion in this time since none of measurements were performed regarding this matter. It had been believed that the *parA* gene is the unique to Cfv [18, 19]; however, motility genes were recently identified, suggesting the possibility of the gene's horizontal transport [19, 20], but this needs to be further investigated. In other hand, if the *parA* gene was transferred to other *Campylobacter* strains we would probably have more positives detected by real-time PCR than by culture. In addition, this potential source of error was equally distributed between all compared protocols.

Even though the magnetic bead-based protocol was the most sensitive in our study, it required more time and resources to perform than heat lysis protocols, and the specificity of all these methods was similarly high. The easiest to perform heat lysis protocol did not significantly differ from the magnetic bead-based isolation method in either sensitivity or specificity and can be potentially utilized for vaginal mucus.

In the chapter 2 the biggest number of positive samples was detected with selective culture of bovine vaginal mucus, when colonies, consistent with *C. fetus* morphology, were subjected to conventional PCR, using MG3F/MG4R and VenSF/VenSR primers. Therefore, this method was chosen for testing vaginal mucus samples in the vaccine trial, described in the chapter 3.

In the second study (Chapter 3) the pregnancy and infection rates between heifers, vaccinated with a polyvalent vaccine containing *C. fetus* antigen, and those, vaccinated with a comparable polyvalent product that did not contain *C. fetus* antigen heifers were compared. Heifers were paired by body weight and then randomized within each pair into treatment or control group. Animals of treatment and control groups expressed estrus and were adequately naturally challenged. There was no significant difference detected by treatment groups in the percentage of heifers that became infected with CfV, the proportion of heifers that became pregnant, and the difference in days to pregnancy. However, serum concentration of *C. fetus* antibodies, detected by ELISA, was significantly higher in treatment group compare to control animals.

Our visual heat detection and chin-ball markers results suggested that all heifers expressed estrus during the first 12 days and were mounted by bulls at least once by the day 17 of exposure. This was equally distributed between two treatment groups. There was also no difference by treatment in the time to expressing the first estrus and first bull mounting.

In this study we used two bulls at the same time of exposure for 63 days. The bulls had satisfactory breeding soundness exams [21]. One of the bulls demonstrated significantly higher breeding activity, compare to another, in the first 12 days. When this more dominant bull was replaced due to a toe abscess, there was no difference in breeding activity between two

remaining bulls from days 13 to 21. However, every bull had similar breeding activity in both treatment groups during the study.

The chin-ball marker results and high culture-positive rate for Cfv from vaginal mucus in both treatment groups suggest adequate challenge. One of the bulls was cultured positive for Cfv only once during the study, which might present a concern about adequate challenge of heifers. However, our chin-ball markers results demonstrated that all heifers that were mounted by that bull were also mounted by other bulls. Overall, 90% of heifers were mounted by more than one bull during the exposure. Those 10% of heifers, mounted by only one bull, were cultured positive several times during the study, also suggesting adequate challenge.

When heifers, mounted by different bulls, were compared in the rate of cultured positive for Cfv at least once during the study, it was similarly high. Heifers were cultured same median number times positive for Cfv during the trial, no matter by which bull they were mounted. Pregnancy rate was low in both treatment groups, compare to be expected in beef heifers under the similar conditions, and was consistent with a reported natural BGC outbreak in western Canada [22].

Overall, the infection challenge was achieved as a total of 86% of heifers, cultured positive for Cfv at least once during the study, and there was no significant difference between treatment groups. When this comparison was performed, the 95% confidence interval (CI) of odd ratio (OR) was of huge range, suggesting the possibility of issue with study power. However, all other 95% CIs of ORs in this study were within less broad ranges. It would be smart though to increase sample size in future related studies.

The highest proportion of vaginal mucus samples with Cfv isolated in our study occurred in weeks 4–6, which differed from weeks 7–15 in another similarly organized trial [22], most likely

because half of the animals in another study were additionally challenged at the day 39 of breeding season by an intravaginal instillation of Cfv [23]. Control groups of both studies demonstrated similar proportion of cultured positive in weeks 4–6.

The uterine secretions are characterized by greater concentrations of IgG than IgA. In some animal models vaginal or nasal application of antigens resulted in optimal uterine response, but this has not been established for cattle [reviewed by 5].

The efficacy of vaccination depends on adjuvants used. The majority of vaccines against *C. fetus* have been prepared with oil or aluminum hydroxide adjuvants [6, 7, 24, 25]. Alum was able to induce a good antibody response, but did not stimulate enough cellular immunity [26] and was not effective when administered, for example, orally or intra-nasally [27, 28]. Oil adjuvants stimulated strong antibody and cell-mediated responses but might cause an injection site granuloma [6, 7, 24]; however, newer oil containing adjuvants have been much improved [20, 26]. For instance, monophosphoryl lipid, isolated from lipopolysaccharide of *Salmonella minnesota* [29], acted on TLR4 and TLR2 [30], did not have inherent toxicity [29] and could be used on mucosal membranes [31].

In our study, pregnancy rate was low and there was no significant difference between treatment groups. These results were in agreement with another study wherein heifers were vaccinated parentally on weeks 6 and 3 before breeding, with a polyvalent vaccine containing Cff and Cfv, and subsequently served by an infected bull for 60 days [25]. However, there was a different study, where the experimental vaccine in oleo adjuvant, containing local strains of *T. foetus* and *C. fetus*, yielded superior protection to the heifers exposed to infected bulls for 90 days [23].

When considering the pregnancy rates in our study, it is important to recognize that the heifers remained exposed to the bulls long enough to get pregnant, abort and become pregnant again. Half of heifers, pregnant at the end of the study, had aborted earlier. We cannot say whether our MAT titers showed improved immunity after abortion since a low number of titer-positives was detected by MAT throughout the study. However, the ELISA results allow to conclude that there was no significant difference in serum concentration of IgG to *C. fetus* between heifers that aborted during the study and those that not. Extending the length of bull exposure has likely improved the overall pregnancy rates in this study, but that might happen in a natural outbreak as well. The time to first detected pregnancy did not differ by treatment. Therefore, vaccination against BGC with polyvalent product did not significantly shorten time to conception in our study.

Most of the disagreements between ultrasound and serum PSPB were in the first weeks of the trial since early pregnancies could cause an increased PSPB concentration but be missed by ultrasound. PSPB has been reported to detect pregnancy about a week earlier than ultrasound [32, 33]. The PSPB level remains elevated for certain period of time after pregnancy loss; however, we used ultrasound to detect pregnancies a couple of weeks later, and only in two cows once elevated PSPB at the beginning wasn't consequently confirmed by ultrasound. In addition, such error could be equally distributed in two groups. More than half of heifers in this study were never detected pregnant; whether they were actually never pregnant or just had early embryonic loss is questionable. It is often cited that CfV results in pregnancy loss at 60–75 days [34, 35], but based on earlier methods of detection, losses could occur earlier. In our study about 90% of abortions happened before day 35 of gestation and abortion rate did not differ significantly by treatment.

Very few positive titers in serum samples from week 2 and 8 of exposure and 10 weeks after exposure were detected during our study, using the MAT method, suggesting no significant differences by week or treatment. After systemic immunization IgG and IgM antibodies are commonly detectable in serum, while IgA and IgG1 are transported across the epithelium into the genital secretions [36]. ELISA has been suggested as the best monitoring test for systemic antibodies against *Campylobacter* spp [37]. In our study significant rise in antibody titers were detected by ELISA in treatment group by week 4 after vaccination right before heifers were exposed to bulls. During the exposure period antibody titers value in the animals of treatment group were significantly higher than of control groups. The commercial vaccine was able to induce antibody response in our study. However, even though the antigenic response in treatment groups was statistically higher, it was probably not as high as it could be when local antigen be used.

The lack of significant antigenic response in our study could be due to antigenic difference between the local strain of the pathogen and that what was used in the commercial vaccine. In another study, a much higher rise in antibodies occurred after utilizing a local strain based vaccine than a commercial vaccine [38]. Alum generally induces an immunity of shorter duration than an oil adjuvant vaccine [38]. Using oleo adjuvant can induce significant antibody response, detectable by *C. fetus*-IgG ELISA test [23]. Therefore, it could be suggested that the adjuvant and/or antigen type of vaccine could cause poor antibody response in our study.

4.2. FUTURE RESEARCH

In our study some modifications were done to the existing heat lysis isolation protocol. All compared isolation methods had low sensitivity in relation to culture and one of possible

explanations is freeze-thaw process of the samples in our study. Rapid freezing results in intracellular ice crystal formation, whereas slow cooling allows water to leach out, reducing crystal formation but leading to osmotic pressure imbalance. Either rapid or slow cooling causes cell membranes to rupture. Ice crystal-induced damage to organelle structures leads to activation of rescue systems, associated with energy generation that results in production of free radicals and oxidative stress to DNA [reviewed by 39]. There are intracellular and extracellular cryoprotectants that can be added to samples. Intracellular agents prevent the formation of ice crystals and extracellular agents reduce the hyperosmotic effect during freezing [reviewed by 39]. Therefore, further testing of the DNA isolation methods should be performed utilizing cryoprotectants or on fresh samples.

The efficacy of vaccination depends on the antigen type and the adjuvant used. Antigenic difference between local and international strains may have caused the lack of vaccine effectiveness [40] and explained the significant rise of antibodies after utilizing the local strains in the vaccine [23] in one study, which was not the case in our study. Aluminum hydroxide adjuvants [6, 7, 24, 25] are able to induce a good antibody response but did not stimulate enough cellular immunity [26] and are not effective for mucosal vaccination [28]. Oil adjuvants stimulate strong antibody and cell-mediated responses but may cause an injection site granuloma [6, 7, 24]; however, newer oil containing adjuvants are much improved [20, 26]. It was suggested that alum generally induces an immunity of shorter duration than an oil adjuvant [38], and an oleo adjuvant can induce significant antibody response, detectable by a *C. fetus*-IgG ELISA test [23]. Therefore, future vaccine trials should be focused on the development and assessment of the vaccine, based on a local strain of Cfv antigen and a newer oil containing adjuvant.

4.3. GENERAL CONCLUSIONS

The heat lysis isolation method, previously successfully utilized for bovine preputial samples, did not demonstrate satisfactory real-time PCR results for vaginal mucus. All compared sample preparation protocols were highly specific, but showed relatively low sensitivity; repeated sample freezing-thawing could be a possible reason. Heat lysis isolation of DNA directly from a CVM sample, skipping all dilution steps, is a simple technique that demonstrated relatively higher sensitivity than other compared heat lysis protocols. The magnetic bead-based extraction protocol is tended to be even more sensitive, but time and resource consuming. Finally, these two methods should be further compared on fresh CVM samples.

Vaccinating heifers with a polyvalent commercial vaccine, containing *C. fetus* antigen, according to the label did not significantly reduce infection rates or improve pregnancy when exposed to Cfv-infected bulls. Antigenic difference between local and international strains may have caused the lack of both significant immunogenic response and vaccine effectiveness. The most effective vaccines are prepared with oil adjuvants; they stimulate strong antibody and cell-mediated responses, and newer adjuvants of such types are much improved and do not cause side effects. Therefore, the vaccine, prepared with local Cfv strains and based on newer oil adjuvants should be created and evaluated.

4.4. REFERENCES

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